NOV 25 1921

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB W. J. V. OSTERHOUT

VOLUME IV, NO. 2 NOVEMBER 20, 1921

PUBLISHED BIMONTHLY

AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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THE NATURE OF FOVEAL DARK ADAPTATION.*

By SELIG HECHT.†

(*From the Marine Biological Laboratory, Woods Hole.*)

(Received for publication, August 15, 1921.)

I.

In a previous paper (Hecht, 1919-20, *c*) on the dark adaptation of the human eye, I attempted an analysis of the existing data on peripheral dark adaptation for the purpose of securing some hint of the physicochemical basis of visual reception. Early in the course of that analysis it became apparent that, aside from the deficiencies in the data themselves, there were lacking some rather elementary concepts on which to rest an objective treatment of the data. For example, there existed no notion of the relation between the intensity of the stimulating light and its photochemical effect in the retina. Moreover, it had apparently never been deemed necessary to entertain any ideas with regard to a mechanism with which to link up the variations in the sensitivity of the retina at different times.

Clear concepts of at least these two matters lie at the foundation of any attempt toward a rationally objective interpretation of the sensory behavior of the eye. It became necessary, therefore, to supply these defects in terms of what had fortunately been found in the studies of the photosensory responses of certain invertebrates (Hecht, 1921, *e*). As a result of these assumptions it was possible to analyze the data of dark adaptation of the eye, and to account for the phenomenon in terms of a comparatively simple photochemical mechanism.

The analysis was made on the best available data. These data were concerned with the periphery of the eye only. This condition

* Part of the apparatus used in these experiments was secured by means of a grant from the Elizabeth Thompson Science Fund. To the trustees of this Fund I wish to express my thanks for the grant.

† National Research Fellow in Chemistry.

proved to be of advantage, because it opened the possibility of connecting the findings with what is known of the properties of visual purple. Moreover, the conclusions which were secured pointed to certain crucial experiments to be made with visual purple, thus suggesting a new mode of attack in this line of work (Hecht, 1920-21, *d*).

However, as far as the eye as a whole is concerned, the analysis was incomplete, because it did not include the properties of the fovea. Although the fovea is essentially an instrument for bright vision, and general dark adaptation, a phenomenon of dim vision, it still seems desirable to find out what occurs in the fovea during a stay in the dark. A study of the published data[1] shows that they are to a large extent insufficient and inconsistent, and that they involve some fundamental sources of error. It has therefore become necessary to investigate the matter from the beginning from a new point of view and with a new type of apparatus. I have done this, and the results show in a gratifying fashion that it was worth while. Not only has it been possible to find the facts and regularities, but it has been possible to account for the discrepancies of the results of other workers.

II.

1. There are three major and several minor sources of error which enter into a study of dark adaptation. With but few exceptions previous work on foveal adaptation has been done without much consideration of the major sources, and with but scant attention to the minor ones. It is necessary to consider these possibilities of error carefully.

Measurements of the course of dark adaptation presume an original condition of light adaptation. It would seem obvious that this must be kept constant if the measurements are to have any significance. Still, this necessary starting point has been consistently disregarded, even when its significance has been recognized. We find that light adaptation is secured by such irregular means as reading in a room (*cf.* Dittler and Koike, 1912) or walking in the street (*cf.* Nagel and Schaefer, 1904). In some cases it is noted whether the day is cloudy or bright, but no account is taken of the circumstance.

[1] A review of the literature will be found in the papers by Tschermak (1902), Wölffin (1910), and Dittler and Koike (1912).

A second source of error involves the change from light adaptation to darkness. It is again almost obvious that this change must be clean cut and accurately timed. In almost all of the previously published data this transition has involved the walking or running of the subject from the light-adapting location to the dark room. It follows that no measurements can be made during the variable interval of running or walking. Even if such measurements were possible, they would be of little significance for a study of the process, because one could not tell when dark adaptation actually began.

The third source of error involves the movements of the iris. At least two distinct things happen when a light-adapted eye is removed to darkness: the pupil dilates and the retina increases in sensitivity. In order that the data may be made to mean something, it is necessary to isolate the separate effects of these two factors.

The means which I used to study foveal adaptation have taken into account all these three sources of error. In addition I have considered certain precautions to limit the measurements to the foveal region. The entire procedure takes place in a large dark room. A constant condition of light adaptation is secured by having the subject look from a fixed distance at a brightly illuminated screen for a given interval. The change from bright illumination to complete darkness is made by shutting off the artificial source of illumination. The result is a clean cut transition. Simultaneously with this change, the subject is not even required to leave his seat. He merely raises his head 5 cm. when the lights are turned off, and is at once ready for measurement.

In the preliminary experiments I attempted to take care of the changes in the iris by the use of an artificial pupil. For several reasons I abandoned this method. First, it was rather clumsy, and proved to be a stumbling block in the accomplishment of a rapid change from the position of light adaptation to that in which the measurements were made. Second, the recent excellent experiments of Reeves (1918) made it possible to correct for pupil variation. And third, in consequence of the feasibility of this correction, it seemed desirable to secure the gross results. This would enable one to make comparisons with previous investigations, and would also give a correct notion of the total change in the eye as a whole, which is itself of some interest.

In order to confine the experiments to central vision, I utilized its characteristic perception of form and color. The object to be looked at was a red cross. The form was secured by a cross-shaped opening of such size that when looked at directly its image fell well within the rod-free area of the macula. The color was secured by a red filter. The measurements were determinations of the minimum illumination at which the subject could clearly see the object both as a cross and as a red one.

2. The essential parts of the assembled apparatus are given in Fig. 1, which shows three views of the arrangements. The screen for light adaptation is marked *N*. It is a closely woven linen screen similar to those used for white window shades. It is illuminated from behind by two Mazda lamps, *L*, 150 watts and 115 volts, backed by polished reflectors, and set 60 cm. from the screen. The switch for turning the lights on and off is in the hands of the experimenter. The subject looks at the region of the screen lying between the two lights; that is, between the two dashed circles in the front view of Fig. 1. The brightness of this field is 90 millilamberts, which is equivalent to an illumination of 0.028 candles per square centimeter of screen.

The construction of the apparatus for measuring the minimum intensity at which the red cross becomes visible is shown in partial section in the side view of Fig. 1. At *C* there is a sheet of opal glass. In contact with it, on the side facing the observer, is a Wratten Filter No. 70. This limits the transmission to the rays beyond 650 $\mu\mu$, and thus insures the participation of the cones only. In front of the filter there is the opening already referred to. It is a cross-shaped opening in an opaque piece of cardboard. It is 24 mm. in diameter, each arm of the cross being 8×8 mm. The area of the cross is thus 320 sq.mm. The observer's eyes are placed next to the opening in the viewing box, above *P*. The appearance of this opening is seen in the front view. The distance from the eye to the cross is 55 cm. The cross therefore gives an image 0.65 mm. in diameter, which is about two-thirds the diameter of the rod-free area.

The illumination of the red cross is secured by means of a small microscope lamp situated in a lamp house, and properly surrounded by black screens. It is a 15 watt, 115 volt, concentrated filament, Mazda lamp, and may be considered as a point source for our purposes.

It rests on a movable carriage which rides freely along the bottom of the long rectangular blackened box shown in the figure. The carriage is only slightly narrower than the box, so that a proper and continuous centering of the lamp is insured. The light from the lamp falls on the opal glass, which diffuses it. It then passes through the filter,

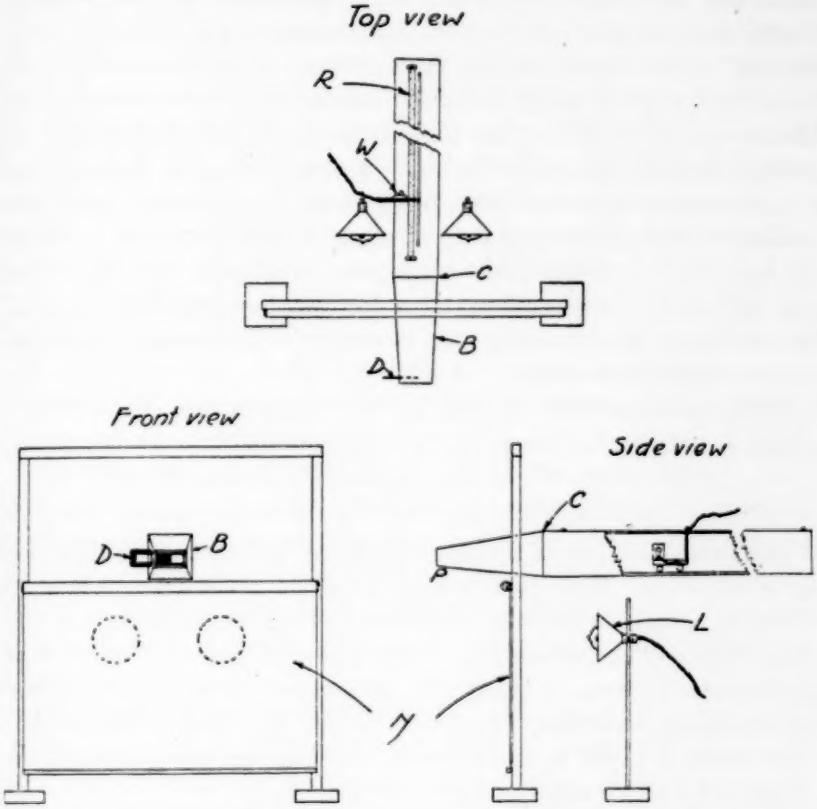

FIG. 1. Diagram of apparatus to secure constant light adaptation, and to measure the course of dark adaptation. An explanation is given in the text.

and goes to the eye. The illumination through the red cross may be varied by changing the distance of the lamp from the diffusing plate of opal glass. The intensity of the illumination emitted by the red cross is measured for a given position of the lamp, and the intensity at any other position calculated on the inverse square law. In these

experiments the lamp was always farther than 30 cm. from the opal glass; in this way a uniform illumination of the cross was assured.

The wires for the lamp run through a brass tube. This is rigidly set in the lamp carriage, and projects through a slit in the upper wall of the blackened box. The brass tube then turns a sharp right angle, and lies about a millimeter above the top of the box perpendicular to the long axis of the box, as shown in the top view of Fig. 1. Because of the rigidity of the construction, the lamp carriage can be easily moved by pushing on the outside arm of the brass tube. Moreover, the location of the lamp is in this way indicated by the position of the horizontal brass arm. An exact record of this position is made in the dark by running a pencil along the edge of the arm, and making a mark on the long strip of paper *R* which is stretched along the top of the box beneath the brass arm. The position of the brass arm indicative of a given location of the lamp is carefully measured. Its subsequent locations may then be read off with a meter stick from the established zero point.

The lamp is controlled by the experimenter by means of a noiseless switch *W* attached to the edge of the protruding brass arm.

III.

The actual manner of making a series of measurements deserves some attention. With each subject I spent a preliminary period of about 30 minutes explaining the nature of the experiments and the construction of the apparatus. Aside from putting the subject into an interested frame of mind, this preliminary period serves as an experimental precaution. It allows time for the effects of the outside light, which is variable, to disappear. The starting point is therefore the same for all the experiments.

The subject is seated in a comfortable chair, the height of which is so adjusted that the eyes of the particular subject can look comfortably into the opening of the viewing box *B*. He then lowers his head, rests his forehead against the edge *P* of the box, and looks at the light-adapting screen. The subject keeps himself in a relaxed condition. To facilitate this, the chair is so placed as to give full support to the subject's back. Also, the edges of the viewing box are padded with black felt. 5 minutes are allowed for light

adaptation. Measurements made with regard to this point show that the process is accomplished in much less time (*cf.* Lohmann, 1906), but this interval is chosen to cover all contingencies. Near the end of the time the subject is given notice, and at the proper moment, the experimenter turns out the bright lights. The subject raises his head at once, and looks into the viewing box.

A few preliminary experiments showed at once that the rate of dark adaptation of the fovea is exceedingly rapid during the first minute of darkness. To make frequent and rapid measurements during this short interval is out of the question. Aside from the technical difficulties, there is the fact that repeated tests of the visual threshold must disturb the process of adaptation. The failure to appreciate this source of error has vitiated many of the experiments of Piper (1903) on peripheral adaptation, and nearly all of the results of Wölfflin (1910) on foveal adaptation. An ideal method would be to make but one reading following each light adaptation. This however would weary the subject, even if it did not fatigue the eye, which is not at all certain. The method finally adopted is a compromise between these two extremes.

Only one eye is used at a time in making the measurements. The eye to be used is under the control of the experimenter, who determines it by moving a slide *D* near the ocular end of the viewing box (Fig. 1). The subject acts as if he were looking with both eyes. Indeed, many of the subjects were unaware of which eye they were using, or that they were using only one eye; a few were able to distinguish them easily.

Before the bright lights are turned off, the small lamp is set at a given distance from the opal glass. When the lights are turned off, the subject looks in and announces the moment he sees the red cross. The time is taken by the experimenter with a stop-watch. The bright lights are turned on, the subject becomes light-adapted again, and the process is repeated with the other eye; this time the light has been placed nearer or farther depending upon the time it took the subject to see the cross. In this way after three or four trials the distance is determined at which the subject can see the red cross almost as soon as he looks into the box, immediately after the room has been darkened. The rate of dark adaptation at the beginning is so rapid

that it is impossible to measure this point accurately. The difficulty lies not so much with the determination of the intensity, as with the measurement of the exact time of dark adaptation. Half a second makes an enormous difference in the threshold, as will presently be apparent. However, a rough idea of the order of magnitude of the threshold after about 2 seconds dark adaptation can be secured in this way, and as such it is valuable.

All this time the subject has gained practice in locating the red cross. The experiment then begins in earnest. After the subject has again been light-adapted, the measuring lamp is set at such a place that the subject will see it after about 5 seconds in the dark. The slide is set for observation with the left eye, and the subject looks in as usual and announces when the red cross appears. The time is taken with a stop watch, the measuring lamp is turned out, and the position of the lamp marked on the strip of paper. The subject withdraws his head from the viewing box, and sits comfortably in the dark.

Readings of the stop-watch and observations of the time in the dark are made by a very dim light which is carefully screened from the subject. This dim light is turned on momentarily when it is needed by means of a spring contact controlled by the experimenter's foot. Repeated tests on this point have never revealed an instance when the subject was able to notice this illumination.

The slide in the viewing box is now adjusted for the right eye. The lamp is moved far back in the long box, and 2 minutes after the beginning of dark adaptation, the subject is requested to look into the viewing box. The measuring lamp is then turned on and brought nearer and nearer the cross opening at a rate of about 3 cm. per second. The subject then announces the moment he sees the red cross. The time is taken with a stop-watch which was started *exactly* 2 minutes after dark adaptation had begun. The lamp is turned off, its position is marked and numbered on the recording paper, and the subject is told to sit back comfortably in the dark. 6 minutes after the beginning of dark adaptation, the slide is set for the left eye, and a measurement made as in the last instance. The process is then repeated after 10 minutes of dark adaptation, this time with the slide set for the right eye. 15 minutes after the beginning of dark adaptation, a reading is made with the left eye again; and after 20 minutes adaptation,

with the right eye. It will be seen that observations with the same eye are made never less than 6 minutes apart. In the other three instances the interval is greater than that, being 8, 9, and 10 minutes, respectively.

After the last observation the bright lights are turned on, and the subject again becomes light-adapted for 5 minutes. The entire process described in the last three paragraphs is then repeated, except that the first observation is made with the right eye, the others following in alternating order. In this way an independent set of determinations is secured for each eye, reaching from the beginning to the end of dark adaptation. These six pairs of duplicate points are, however, not sufficient to map out accurately the entire course of dark adaptation. Therefore intermediate points are secured in the following way.

The subject is again light-adapted. The lights are turned off, and a determination is made with the left eye after about 30 seconds dark adaptation. The lamp is brought closer and closer in the usual manner until the subject announces the appearance of the red cross. The time is taken and the distance recorded. The subject rests in the dark, and after 3 minutes in the dark a measurement is made, this time with the slide set for the right eye. The bright lights are then turned on, the subject becomes light-adapted, and the 2 measurements are repeated, this time first with right eye for 30 seconds adaptation, and then with the left eye after 3 minutes dark adaptation.

It is not necessary to detail the method any further. Suffice it to say that two sets of ten determinations are secured, a set for each eye. The whole procedure may be shown in Table I, which gives the details of a single experiment. The table shows first, the order in which the observations are made; second, the period extending from the time when the bright lights were turned off, to the time when adaptation was announced; and third, the distance which the measuring lamp had to be brought before the subject announced that she saw a red cross. Each horizontal line in the body of the table indicates 5 minutes light adaptation. Thus, between two horizontal lines are the readings made during a single period of dark adaptation. An entire experiment takes about an hour and a half.

An observation is never verified immediately. Once the subject has announced that he sees the red cross, the lamp is at once turned off. If an observation is questioned either by the subject or by the experimenter, it is repeated later after a fresh light adaptation, and the subject is unaware of its repetition. The practice of verifying a reading within a second or so involves a number of fresh sources

TABLE I.

Record of a Single Experiment on Dark Adaptation of Fovea.

Subject, M. G. ♀

Left eye.			Right eye.		
Order No.	Duration of adaptation.	Distance.	Order No.	Duration of adaptation.	Distance.
	min.: sec.	*mm.*		*min.: sec.*	*mm.*
1	:06	352	2	2:15	1060
3	6:12	1410	4	10:15	1600
5	15:17	1345	6	20:10	1435
8	2:10	1100	7	:05	352
10	10:16	1355	9	6:12	1355
12	20:13	1480	11	15:10	1460
13	:32	635	14	3:12	1345
16	3:11	1235	15	:30	595
17	:16	470	18	1:01	880
20	1:02	805	19	:17	495

Horizontal lines between measurements indicate a period of 5 minutes light adaptation.

of error such as retinal fatigue and refractory period which are bound to influence the final result (*cf.* Cobb, 1919, p. 437). I, therefore, consistently avoided such a method.

IV.

1. After several preliminary experiments, I made a final set of measurements with fifteen subjects. Of these, eight were men and seven women. Their ages varied between 28 and 35 years. They were all of more than ordinary intelligence, all but one having been

university trained. Most of them were teachers; the rest were professional people acquainted with scientific matters. They were all interested in the experiments, and did all they could to follow instructions in the method of making observations.

The nature of the data which I secured with these people is well illustrated in Fig. 2, which gives the results of two experiments on the same subject made four days apart. Each point represents a single reading with one eye in the manner described. The ordinates

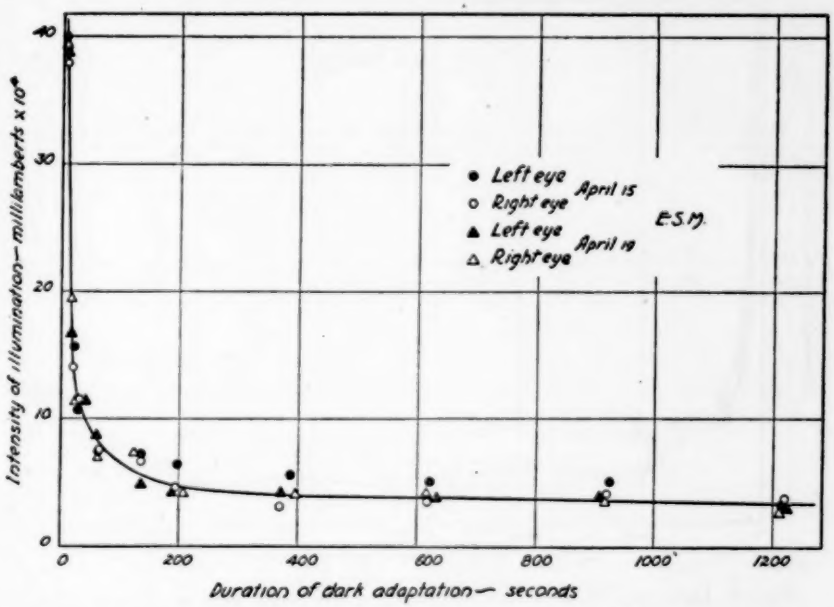

FIG. 2. Results of two experiments made 4 days apart with the same subject. The points are single readings as explained in the insert.

record the illumination emitted by the red cross when it just becomes visible. The data are not corrected for changes in the pupil area. The figure shows very clearly that the experiments were conducted under conditions that give reproducible results. I made a number of these duplicate sets on several individuals, and all show the same degree of coincidence in the position of the points.

In the results as a whole there is a certain amount of variation from individual to individual. One subject may be consistently

less or more sensitive than another. However, the shape of the curve of dark adaptation remains the same, and the degree of variation is not much more than would be expected from inspection of the points in Fig. 2. To show this, I have plotted in Fig. 3 the results of the first five subjects. The other ten subjects give exactly the same kind of data. Each point in Fig. 3 is the average of two readings, one for the left eye and one for the right. It is apparent

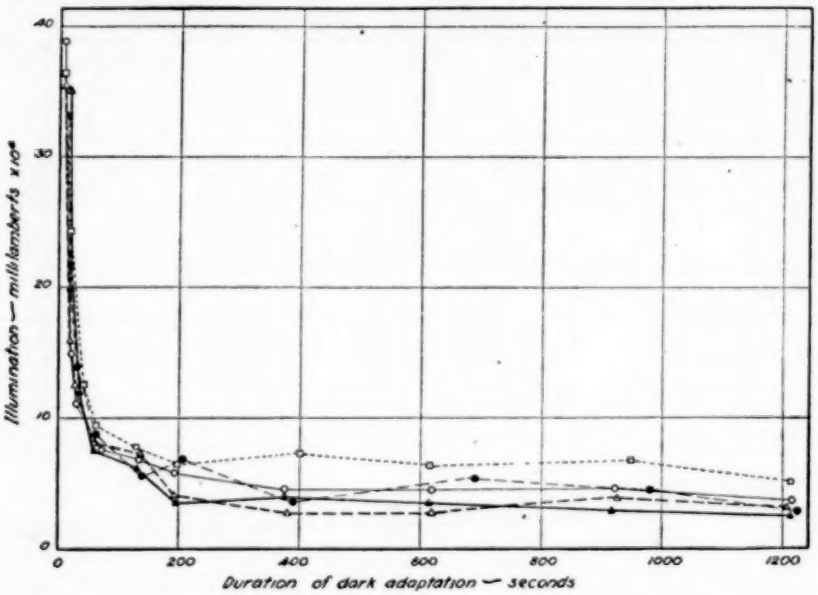

FIG. 3. Data for the first five subjects. Each point is the average of two readings, one for the left, and one for the right eye.

that the experiments are reproducible not only in a given individual, but in a group of individuals as well. In presenting the complete data I have therefore combined the measurements of the fifteen subjects, thus getting an average of thirty determinations of each of the ten points on the curve of dark adaptation. These average results are given in Fig. 4. It will be seen that the shape of the curve and the location of the average points are similar to those shown for the single points in Figs. 2 and 3.

2. One thing stands out prominently in all these figures. It is that the dark adaptation of the eye, as measured by foveal vision, begins immediately, and proceeds at a precipitous rate for the first 30 seconds. The data in Figs. 2, 3, and 4 begin to record the events which happen after the first 7 seconds in the dark. This is because things happen too fast for accurate measurement before the first 7 seconds. As noted in a previous section, however, I have made some observations of the threshold after 2 seconds of dark adaptation for

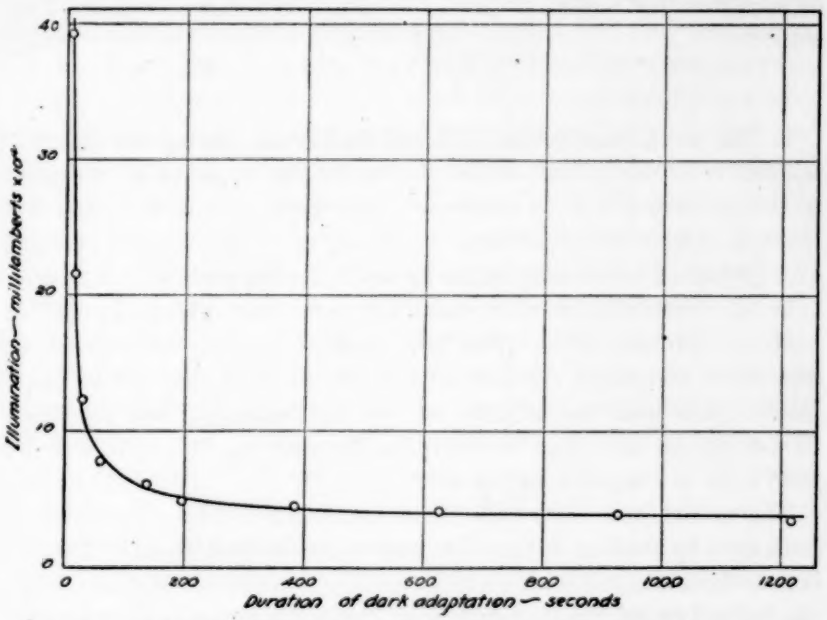

FIG. 4. Data for all fifteen subjects. Each point is the average of thirty measurements.

the purpose of securing an idea of the order of its magnitude. An average of twenty measurements gives this threshold a value of 140×10^{-4} millilamberts. From Fig. 4 it can be seen that the threshold after 7 seconds of dark adaptation is about 40×10^{-4}ml. After 30 seconds in the dark the threshold drops to about 12×10^{-4}ml. Because of the nature of the curve of dark adaptation, as described in a later section, it is possible to extrapolate backwards and find what the value of the threshold is at zero seconds dark adaptation. This turns out to be

454×10^{-4} ml. The threshold thus drops in the first 30 *seconds* to about 3 per cent of its initial value.

During the next 20 *minutes* the threshold further decreases, but only to about 3×10^{-4} ml., and remains there. It is therefore clear that the change which takes place after the first 30 seconds is comparatively insignificant in the light of the first rush of events. There is, however, no sudden point at which the process of adaptation begins to slow down. It slows down from the moment it begins, and as can be seen from the figures presented so far, its course is continuous and smooth.

V.

1. The precipitous course of foveal adaptation during the first few seconds is at the bottom of the curious results obtained by previous workers. It will not be irrelevant to consider in some detail the better and more recent of these investigations. Inouye and Oinuma (1911) studied foveal adaptation by the following method. A double tube was constructed through which the two eyes could look simultaneously at different fields. The brightness of the two fields could be separately controlled by a rotating sector of black and white cardboard. The idea was to keep one eye light-adapted, and the other dark-adapted, and then to vary the illumination in one tube until both eyes saw equally bright fields.

The actual procedure calls for a preliminary light adaptation of both eyes by reading in the laboratory near the dark room. One eye is then dark-adapted with a black bandage. After the proper interval the subject rushes into the dark room, tears the bandage from his eye, and looks through the two tubes. The sector wheel has been set, and the subject compares the brightness of the two fields. After repeated trials for the same amount of dark adaptation, the position of the sector is found which results in apparent equality of the two fields. The entire process is then repeated for different amounts of dark adaptation. These experiments were repeated by Dittler and Koike (1912), who used smoked glasses instead of a sector wheel with which to reduce the illumination of the dark-adapted eye.

The results secured by these two sets of investigators show a leisurely kind of dark adaptation for the fovea, Dittler and Koike's

data showing much the slower rate. For example, after 3 minutes adaptation the eye requires 22.5 per cent of the illumination of the light-adapted eye in order that both see the same brightness. After 30 minutes adaptation the ratio of light to dark-adapted eye is as 100 to 8. This represents total dark adaptation.

The explanation for this leisurely adaptation lies in the extreme rapidity with which adaptation actually proceeds during the first few seconds. Consider Dittler and Koike's method. One eye is light-adapted while the other is dark-adapted, and the standard of measurement is the light-adapted one. To get into the dark room takes a few seconds; to get to the apparatus takes a few more seconds; to remove the bandage, to accommodate, and to make a judgment also take a few seconds. During part of this time the light-adapted eye is in the dark, and as Fig. 4 shows, it is very rapidly becoming dark-adapted. When the comparison is being made, the light-adapted eye is no longer light-adapted. Its threshold is much lower than it was a few seconds ago. As a standard of comparison it is entirely too low, the situation being the same as if the measurement of dark adaptation were begun after the first few seconds have elapsed. This, together with the fact that the degree of light adaptation is low to begin with, could make the course of adaptation correspond with that portion of the curve in Fig. 4 which lies below, say, the middle of the scale of ordinates. This condition accounts for the apparent slowness of the process and for its small extent, both of which are characteristic of these and of the older investigations.

2. Considered from this point of view, these older results constitute a significant check on the validity of the experiments presented in the present paper. My measurements were made with red light, and it might perhaps be thought that the course of dark adaptation would be different if the experiments had been conducted with white light.

To meet this criticism I made a few experiments on my own eyes, using red light at one time and white light at another. Although the actual threshold illumination is not the same in the two cases, the rate of adaptation is the same. This matter has already been considered by Nagel and Schaefer (1904, p. 283), who came to the same conclusion. They used red, blue, and green lights and found that the same order of change is secured with all three colors. In other words, the Purkinje phenomenon does not exist in the fovea.

Moreover, the experiments which I have just reviewed, though differing in accuracy from mine, are indeed roughly comparable to them when properly evaluated, as I have pointed out above. They show, for example, that foveal adaptation practically ceases after 10 minutes in the dark. In addition, if the value of the light-adapted eye as a standard be tripled or quadrupled, as it must at least be because of the initial speed of adaptation, the final degree of dark adaptation represents about 1 per cent of the condition of the light-adapted eye. This is practically the same value which is found in the present experiments. Calling the initial threshold 454×10^{-4} ml., the final threshold of 3.4×10^{-4} ml. represents a drop to 0.8 per cent. Therefore the results with red light show the same course of adaptation as experiments with any other light. A careful analysis of our present data will thus be of significance for the general problem of adaptation and foveal vision.

VI.

The data as they stand include the results of two phenomena: the movement of the iris, and the change in the fovea. In order to isolate the effects due to the foveal changes alone, it will be necessary to correct the data for variation in the pupil size. If the pupil area did not vary, the energy received by the retina would depend on the intensity of the illumination. On the other hand, if the intensity were to remain constant, the energy transmitted to the retina would be directly proportional to the varying area of the pupil. It then follows that since both intensity and pupil area vary, the energy received by the retina is proportional to the product of the intensity and the area.

We have measured the intensities; it is therefore necessary to introduce the data for the area of the pupil. These can be supplied from the experiments made by Reeves (1918). The experimental situations in the two cases are comparable. Reeves determined the changes in the pupillary diameter in darkness following adaptation to artificial illumination of about 100 ml. In my experiments the light adaptation field had a brightness of 90 ml. These illuminations result in practically the maximum effect in constricting the pupil.

In Fig. 5 are given the results calculated from the diameter values in Table IV of Reeves' paper. From the smoothed curve it is simple to find the pupil areas corresponding to the points plotted in Fig. 4. It is not necessary to correct for the effect of the cornea on the apparent size of the pupil, because we are interested in the relative variation in the pupil area. Calling unity the area of the pupil after 7.1 seconds dark adaptation, the first point in Fig. 4, I have computed the relative areas of the pupil for the different points in Fig. 4. These values are given in Table II.

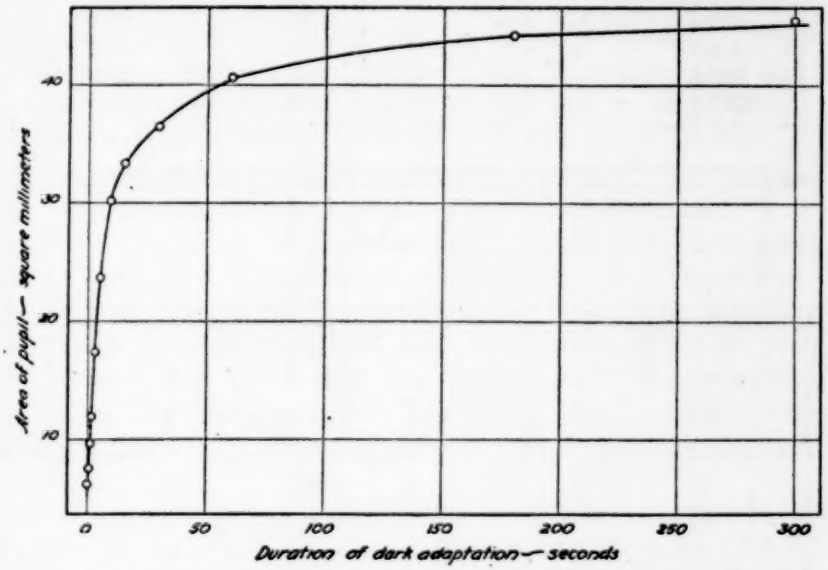

FIG. 5. Change in the area of the pupil during dark adaptation. The data are computed from the diameters given in Table IV of Reeves' (1918) paper.

In the last column of the same table are given the values of the products of the measured intensity into the relative area of the pupil. These numbers represent the intensity thresholds at different times in the dark on the assumption that the pupil area has maintained a constant area equal to its value at 7.1 seconds. This point is chosen arbitrarily because it is the first accurately measured point in these experiments. Any other point would have done just as well, because all that we are concerned with are the relative changes at constant

TABLE II.

Relation between Apparent Illumination, Area of Pupil, and Corrected Illumination.

Duration of adaptation.	Observed illumination.	Relative area of pupil.	Corrected illumination.
sec.	*ml.* × 10^4		*ml.* × 10^4
7.1	39.30	1.00	39.30
16.4	21.60	1.19	25.70
29.3	12.30	1.28	15.80
59.5	7.81	1.47	11.50
136.0	6.09	1.56	9.50
196.0	4.78	1.58	7.57
385.0	4.49	1.62	7.28
626.0	4.06	1.63	6.62
925.0	3.86	1.63	6.30
1217.0	3.37	1.63	5.50

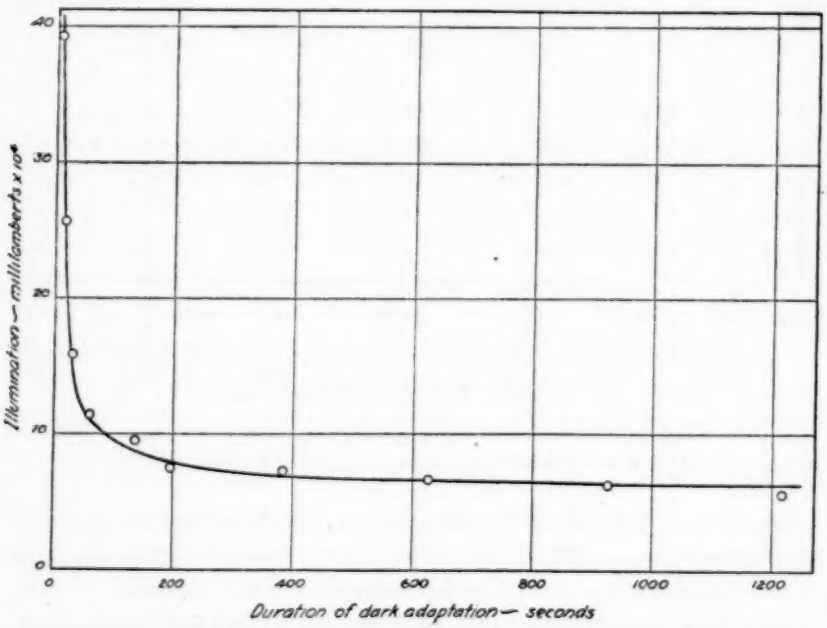

FIG. 6. Dark adaptation of fovea independent of pupil changes. The points are those in Fig. 4 corrected in terms of Fig. 5. They represent the illumination necessary to stimulate the fovea assuming a constant pupil whose area is 27.1 square millimeters. This is the area at 7.1 seconds dark adaptation, and corresponds to the first point in Figs. 4 and 6.

pupil diameter. These results are plotted graphically in Fig. 6, and their values are indicative of the changes which take place in the fovea independent of the movements of the iris. It is to the interpretation of these data that we shall now turn our attention.

VII.

In considering the action of light in vision, it is hardly an assumption to suppose that the first effect of the light is the photochemical decomposition of a sensitive substance. Our measurements represent this effect in terms of the minimum intensity of illumination required to produce a visual sensation at different times in the dark. What is the relation between the intensity of the stimulating light and its objective photochemical effect? In the analysis of peripheral adaptation (Hecht, 1919-20, *b*) I assumed that the photochemical effect is proportional to the logarithm of the intensity. The reasons for this assumption were first, that such a relationship had been found in the clam, *Mya;* and second, that the gamut of change undergone during peripheral adaptation was so enormous that a logarithmic relation is the only one that would bring the data into a form amenable to ordinary comprehension and analysis. These reasons are not imperative. They derive their final justification on the pragmatic ground that the assumption of a logarithmic relation results in the formulation of a simple chemical picture which accounts for the data satisfactorily.

The range of dark adaptation in the fovea is not a tithe of the extent encountered in the periphery. The older data indicate a change from 10,000 to 1 in the minimum intensity necessary for a peripheral effect. Cobb's (1919) more recent experiments, as well as my own unpublished ones, indicate even a greater change than this. The older measurements were faulty in a number of ways, particularly in their neglect of the first few seconds of adaptation. Compared to these changes, those presented here for the fovea are really small in extent. When corrected for constant pupil diameter, the necessary minimum for a response after 2 seconds of foveal adaptation is about 70×10^{-4} ml. The extrapolated value at zero becomes, after pupil correction, 103×10^{-4} ml. After 20 minutes the intensity as given in Fig. 6 is about 6×10^{-4} ml. This range of change represents noth-

ing startling, it being the kind ordinarily encountered in chemical processes. In treating the data further I have therefore assumed that the photochemical action of the light is directly proportional to the intensity. This is a common enough situation in photochemical reactions, and in addition, obtains in the photic sensitivity of *Ciona* (Hecht, 1918–19, *a*).

It follows from Fig. 6 that the quantity of sensitive material decomposed in order to produce a threshold effect in the fovea gradually but consistently decreases during dark adaptation. What is the basis for this variation? In other words, with what chemical or physical entity or process in the fovea can this gradual decrease be associated? I have previously outlined a possible hypothesis to account for this, and because of its success in the study of the responses of *Ciona* and *Mya* as well as of the periphery of the human eye (Hecht, 1921, *e*), I venture to suggest its application in the present circumstance.

Let us consider what might conceivably happen in the fovea. We have accepted the existence of a photosensitive substance. During light adaptation this substance must be decomposed to a considerable extent. Associated with this is a decided increase in the threshold of stimulation. During the succeeding period of dark adaptation there is produced a characteristic decrease in the threshold which proceeds according to a definite pattern. During this time it seems reasonable to suppose that the sensitive material is in some way being formed again, and that its concentration in the sense cells is increasing.

The continuously decreasing threshold cannot be directly associated with this increase in sensitive material. One reason for this is the fact that they vary in the opposite sense. A more cogent reason is the nature of the sensitive material itself. In its essence it must be an inert material so far as the sensory process is concerned. Otherwise it would continually be initiating visual effects. It is only after it has been changed into something else by the light, that it produces its characteristic reaction.

Indeed, it is to this decomposition product that we must look for some objective visualization of the process of dark adaptation. This decomposition product undoubtedly enters into some secondary reaction. I have elsewhere suggested that in *Mya* and *Ciona* it serves to catalyze a second reaction, the end-products of which constitute

the "inner stimulus" for the nerve endings. It is not necessary at present to be specific on this point. It is enough to accept the general idea that the decomposition product is the active agent in the early stage of the formation of the visual impulse. Its effects, catalytic or otherwise, will undoubtedly be a function of its concentration in the sense organ. An increase in this concentration will cause an increase in its effect. It would, therefore, seem a reasonable assumption to make that the increase in concentration necessary to produce a perceptible effect in the secondary reaction is directly proportional to the concentration already present. This is the "compound interest law" which is at the bottom of so many physical and chemical phenomena.

The assumption then is that in order to produce a photosensory effect, the light must cause an increase in photolytic products proportional in extent to the concentration already present in the sense organ. Such an assumption has served successfully in the analysis of the photosensory process in *Mya* and *Ciona* and in the periphery of the human eye. Moreover, in the case of *Ciona* it was possible to put the matter to a critical experimental test, the results of which completely vindicated the assumption (Hecht, 1918-19 *a*, p. 162).

VIII.

In the case of the present data as given in Fig. 6, this assumption plus the one relating intensity and photochemical effect lead to this. The ordinates, representing the intensity of the threshold, are directly proportional to the amount of photolytic products formed by the light. These values are in turn directly proportional to the concentration of photolytic products already present in the sense organ. The curve of dark adaptation of the fovea therefore represents the changes which take place in the concentration of the photochemical decomposition products in the sensory mechanism.

It is apparent that this concentration is decreasing steadily. This decrease can represent one of two possible processes: either the products are diffusing out of the sensory cells, or they are reacting chemically in the formation of some other substance. The nature of the curve in Fig. 6 will be of some aid in deciding between these alternatives.

The relation between the intensity I and the photochemical effect E is linear, and is represented by the equation of a straight line

$$E = k\,I + b$$

The constant k will depend on the units employed; it may therefore be made equal to unity by changing the units. The constant term b represents the intercept on the y axis. In the present case, omitting the factor 10^{-4}, it turns out to have a value of -6.0.

It is then necessary to find what the relation is between the photochemical effect E, representing the concentration of photolytic products, and the time t during which the eye has been in the dark. Calculation shows this relation to be that of a bimolecular chemical reaction

$$k = \frac{1}{at} \times \frac{x}{a - x}$$

in which k has a value of 0.00271.

In the calculation of the data according to the bimolecular isotherm, a is taken as the value of the first point in Fig. 6. This makes a equal to $39.3 - 6.0$, or 33.3 units. Correspondingly, t is taken as zero at 7.1 seconds, and the other values of t are computed accordingly. This avoids the use of any highly uncertain values of the threshold at the first movement of dark adaptation. Once the constant k has been determined in this way, it is possible to calculate the real value of a at $t = 0$. This extrapolation from the experimental results and the above equation gives a a value of 103.2 units. It should be noted that the curve in Fig. 6 is drawn from values calculated from the equation of a bimolecular reaction in which k has the value already given.

Several things follow from the fact that the curve is that of a *bi*molecular reaction. One is that the gradual disappearance of the decomposition products is not a process of diffusion. Under certain conditions the rate of diffusion may be a linear function of time. More normally it is a function of concentration. In the latter event its course is represented by an equation similar to that of a monomolecular reaction. A bimolecular reaction isotherm represents not a process of diffusion, but one of chemical reaction only.

Granting the chemical nature of the process, it is obvious that there exist two products of decomposition which are disappearing from the reaction mixture by combining to form something else. Concomitant with this, in the same sense cells, there is being produced an increasing concentration of photosensitive material. What more reasonable step is there than to assume that it is being formed by the combination of the decomposition products that are disappearing?

We are therefore led to the conclusion that in the photosensory mechanism of the fovea there exists the following arrangement of materials. There is a photosensitive substance S whose rate of photolysis at threshold intensities is a linear function of the intensity. The photolysis of this material results in the formation of two products of decomposition P and A. The concentration of these decomposition products at any moment determines the sensitivity of the sensory mechanism, in that the amount of photolytic action necessary for a threshold, visual effect is directly proportional to the concentration of photolytic products already present. Finally, these decomposition products constantly recombine to form fresh sensitive material, their combination proceeding according to the kinetics of a bimolecular reaction. We have thus the familiar reversible reaction

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A$$

in which the products of decomposition serve as the precursors of the sensitive material.

IX.

It is not necessary at this time to dilate on the simplicity with which such a reversible system accounts for the observed facts (*cf.* Hecht, 1919-20, *c*, p. 514). It will suffice to point out that the "dark" reaction obviously accounts for dark adaptation; that the stationary state, in which the "dark" and light reactions are balanced, takes care of the condition of sensory equilibrium in which the eye has become adapted to a given light intensity; and that the process of light adaptation represents merely the displacement of the stationary state of the reaction to the right, due to the increased action of the light.

It is worth drawing attention to the fidelity with which the calculated curve of Fig. 6 adheres to the observed facts of dark adaptation. For further testimony Table III is presented showing this agreement in the usual manner. Considering all the things which enter into the experiments and into the calculations, the agreement seems striking.

This agreement, however, need not be considered an infallible *experimentum crucis*. It is possible that some other formula might fit the facts just as well. However, the significance which attaches to the agreement is that it follows from a reasoned construction of what may

TABLE III.

Comparison of the Observed Values for the Threshold during Dark Adaptation with Those Calculated in Terms of a Bimolecular Reaction.

Duration of adaptation.	Threshold intensity.	
	Observed.	Calculated.
sec.	*ml.* × 10^4	*ml.* × 10^4
7.1	39.3	(39.3)
16.4	25.7	24.1
29.3	15.8	17.0
59.5	11.5	10.9
136.0	9.5	8.6
196.0	7.6	7.9
385.0	7.3	7.0
626.0	6.6	6.6
925.0	6.3	6.4
1217.0	5.5	6.3

take place in the cone cell of the fovea. The circumstance that a similar type of photochemical mechanism differing in local detail of application serves to account for the photosensitive behavior of *Mya*, and for that of the peripheral retina, heightens the plausibility of the general picture.

With regard to these local differences in the treatment of the general idea there are several interesting points to be considered. I shall indicate just one of these. For the periphery (rods) of the eye the application of the principle of a reversible reaction depends on the assumption of a logarithmic relation between intensity and photo-

chemical effect. For the fovea (cones), however, a similar interpretation depends on a linear relation between intensity and photochemical effect. What constitutes the basis of this difference between rod and cone will be a nice point if it is ever established. I have already suggested (Hecht, 1919-20, *b*) that the logarithmic relation may hinge on some absorption phenomenon associated with the sensory mechanism. However, a detailed treatment of this and other possibilities is not appropriate at this juncture, and will be reserved for a later occasion.

The general idea which I have suggested as underlying all these phenomena of photic sensitivity has an immediate use. In the case of *Mya* it has served as a means of suggesting fresh and crucial experiments that have already contributed materially to its support. In venturing to suggest a similar mechanism for the eye, I hope that it may help to point the direction in which further work may be done. It would seem that by such a method progress may be made in the rather chaotic field of visual physiology. A concrete picture, though it limits itself to the initial events in vision, should serve better than the vague, sweeping theories of which there are already far too many.

SUMMARY.

1. After a discussion of the sources of error involved in the study of dark adaptation, an apparatus and a procedure are described which avoid these errors. The method includes a control of the initial light adaptation, a record of the exact beginning of dark adaptation, and an accurate means of measuring the threshold of the fovea after different intervals in the dark.

2. The results show that dark adaptation of the eye as measured by foveal vision proceeds at a very precipitous rate during the first few seconds, that most of the adaptation takes place during the first 30 seconds, and that the process practically ceases after 10 minutes. These findings explain much of the irregularity of the older data.

3. The changes which correspond to those in the fovea alone are secured by correcting the above results in terms of the movements of the pupil during dark adaptation.

4. On the assumption that the photochemical effect of the light is a linear function of the intensity, it is shown that the dark adaptation

of the fovea itself follows the course of a bimolecular reaction. This is interpreted to mean that there are two photolytic products in the fovea; that they are disappearing because they are recombining to form anew the photosensitive substance of the fovea; and that the concentration of these products of photolysis in the sense cell must be increased by a definite fraction in order to produce a visual effect.

5. It is then suggested that the basis of the initial event in foveal light perception is some mechanism that involves a reversible photochemical reaction of which the "dark" reaction is bimolecular. Dark adaptation follows the "dark" reaction; sensory equilibrium is represented by the stationary state; and light adaptation by the shifting of the stationary state to a fresh point of equilibrium toward the "dark" side of the reaction.

Dr. W. E. Forsythe of the Nela Research Laboratories was kind enough to calibrate the illumination emitted by the red cross and by the light-adapting screen. I take pleasure in thanking him for his courtesy and help in this connection.

I also wish to express my appreciation of the gracious cooperation of my various friends who served as subjects in what must have been a tedious performance. I feel particularly indebted to those who were sufficiently interested and kind to serve as subjects for duplicate and triplicate experiments.

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THE SELECTIVE ABSORPTION OF POTASSIUM BY ANIMAL CELLS.

II. THE CAUSE OF POTASSIUM SELECTION AS INDICATED BY THE ABSORPTION OF RUBIDIUM AND CESIUM.

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(Received for publication, July 9, 1921.)

That the peculiarities of the physiological behavior of potassium may be related to its ionic mobility has been suggested by Bayliss (1). Loeb (2) has referred its peculiarities to its electronic structure; *i.e.*, to the outside ring of electrons and its atomic number. These, in accord with modern views, would determine hydration. Hydration, according to Jones (3), is responsible for the difference in ionic velocities of members of the group of alkali metals. The arrangement of outer electrons and the atomic number then may be regarded as determining the relative ionic mobility. This is the general explanation as set forth by Mines (4) and others as to why ionic mobilities have a complex relationship to ionic volumes. The fact that potassium is generally selected in preference to sodium by a great variety of living cells is especially notable among its several peculiarities. Is there any evidence to show that the difference in the migration velocities of potassium and sodium are concerned in this so called "selective action"?

Among the comparative ionic conductances of the univalent metallic ions, together with the ammonium ion which has similar behavior, we note certain relations. The figures below are taken from Landolt (5) and refer to ionic conductances at 18°C.

Li	Na	NH_4	K	Rb	Cs
33.4	43.5	64	64.6	67.5	68

The interval between Na and NH_4 is much greater than intervals between the others. Lithium and sodium may be regarded as constituting one group, while ammonium, potassium, rubidium, and cesium fall into another. These are all univalent ions so that their absolute velocities of migration show the same relations as their conductances. If, then, the absorption of potassium by cells in preference to sodium is related to comparative ionic mobilities, lithium should behave like sodium, but rubidium and cesium like potassium. In a number of other physiological processes, just such relationships have been shown. Loeb (2) has pointed out how lithium and sodium stand in one group, physiologically speaking, while ammonium, potassium, rubidium, and cesium clearly constitute a distinctly different group. The members of each group show among themselves a number of similarities in physiological behavior, while the effects of the two groups may be regarded, in some ways, as physiologically divergent. Because of the manner in which hydration affects ionic mobilities the migration velocities of the members of this series seem to provide a basis for grouping them in accordance with their physiological behavior more satisfactorily than do other periodic functions, as for example, atomic volumes. We have sought to show whether or not the contrast of physiological properties among this series holds for their intracellular incorporation. We have not yet made a study of lithium. There is much in the literature on the subject to indicate that lithium is capable of penetrating into cells with no greater facility than sodium. The behavior of ammonium ions is obviously without much bearing on the question of potassium selection, since they freely pass in and out of all cells and are to be regarded, in general, as a waste product and not as constituents of cellular architecture. The experiments herein reported go to prove that rubidium and cesium behave like potassium in processes involving incorporation into the physicochemical structure of muscle and other animal cells.

Mendel and Closson (6) found that rubidium injected into cats and dogs was largely stored in muscle tissue. Zwaardemaker (7) and his coworkers in researches on aequiradio activity have shown that rubidium and in some degree cesium can replace potassium in maintaining the heart beat, but whether this means penetration into cells or action at their surfaces it is difficult to say. In the light of

our observations we would conclude that it shows the former to be true even though the effect upon irritability may be at the cell surface. So far as we have been able to read Zwaardemaker's reports in the original we have found no evidence on this aspect of the question and in this connection our results seem of some significance.

EXPERIMENTAL.

We perfused frog muscles with a Ringer solution modified by the replacement of potassium chloride with an aequimolar concentration of rubidium chloride. While both legs were perfused the muscles of one were made to contract by stimulation of the lumbar plexus with maximal tetanizing induction shocks lasting 1 second at 30 second intervals during one-half hour periods with alternating one-half hour periods of complete rest. In one experiment this procedure was continued during 5 hours and was followed by perfusion with an isotonic cane sugar solution during 1½ hours. All the muscles of both legs showed irritability at the end of the experiment. Samples of the gastrocnemius and sartorius of each leg were then decomposed in a mixture of nitric and sulphuric acids. The resulting solutions enabled us to detect rubidium spectroscopically in the muscles of the stimulated leg but not in those of the other. In another similar experiment the muscles of one leg were given nine half-hour work periods, that is, 540 contractions of 1 second each, and were then while resting, perfused during 2 hours with a potassium-free Ringer solution. The muscles of both legs showed good irritability at the end of the experiment. The wet-ashed muscle samples, taken from the gastrocnemius and vastus muscles of each leg, were made up to 10 cc. and examined spectroscopically by Gooch's method for quantitative estimation of rubidium. No trace of rubidium could be detected in the muscles perfused without stimulation. The samples used were 1.27 gm. from the gastrocnemius and 2.37 gm. from the vastus. In the muscles of the stimulated legs, however, we found approximately 0.011 per cent of rubidium. The standard solution for spectroscopic comparison with the muscle material contained 3 per cent of H_2SO_4, 0.075 per cent of KCl, 0.025 per cent of NaCl, and 0.0025 per cent of rubidium added in the form of the carbonate. It was designed to imitate, approximately, the acid, potassium and

sodium content of the solutions of wet-ashed muscle. The samples taken from the stimulated muscles were 1.86 gm. from the gastrocnemius, and 2.03 gm. from the vastus. The standard solution diluted 3.5 times gave the faintest rubidium spectrum possible to detect. The solution from the gastrocnemius behaved similarly when diluted 3 times and the one from the vastus when diluted 3.5 times. A careful uniformity of technique was used in making all the flame tests. This estimation shows 0.011 per cent of rubidium in the gastrocnemius and 0.012 per cent in the vastus. As a matter of fact a proportion of rubidium larger than these figures indicate was present because the muscles were obviously edematous. Rubidium content, in proportion to the dry solids, was not estimated. An amount of rubidium, equivalent to more than 3 per cent of the normal potassium content

TABLE I.

The Absorption and Retention of Cesium by Frog Muscle

Volume of cesium solution used.	Duration of first perfusion.	Volume of potassium-free Ringer solution used.	Duration of second perfusion.	Weights of samples of muscle used for analysis.				Cesium found.	
				Right gastrocnemius.	Right vastus.	Left gastrocnemius.	Left vastus.	Left gastrocnemius.	Left vastus.
cc.	*hrs.*	*cc.*	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
625	7	530	4½	2.80	2.78	2.57	2.37	0.008	0.006
475	6½	500	3½	3.01	2.39	2.55	2.97	0.006	0.006

of frog muscle, was taken up when the muscles made 540 contractions of 1 second each. The retention of rubidium in the muscles during perfusion with a rubidium-free solution and the absence of rubidium from the unstimulated muscles, points to its actual entrance into and incorporation with the cell substance in the same sense that potassium is normally held there. Whether or not it actually replaces potassium has not yet been determined.

Similar experiments were made with cesium chloride, replacing, in equimolar concentration, the potassium chloride of Ringer solution. The data of two such experiments are presented in Table I. The muscles of the left leg of the frog used in each experiment made 420 contractions of 1 second each, while the cesium-containing solution was perfusing through both legs. The muscles of the right leg were meanwhile at rest. At the end of the experiment the muscles of

both legs showed good response to both direct and nerve stimuli; though, to the same stimulus, muscles of the right leg responded, of course, more vigorously than those of the left. Adequate samples of muscles of the right legs (amounts of samples are recorded in the table) showed no trace of cesium in a careful spectrum analysis. Muscles of the left legs showed brilliant cesium spectra, permitting a quantitative estimation. Cesium, then, like rubidium was taken into the muscle substance so as to be retained, in part, during the subsequent perfusion with potassium-free Ringer solution.

To further test the replaceability of potassium by rubidium and cesium young white rats were fed on purified synthetic diets in which salts of rubidium or cesium were substituted for those of potassium as ordinarily used in such diets. The basal diet had the composition shown below.

	per cent		*per cent*
Casein	18	Dried yeast	5
Starch	54	Salts	5
Butter fat	18		

In the experiments with rubidium each 5 gm. of salt mixture contained approximately:

	gm.		*gm.*
$MgSO_4 \cdot 7H_2O$	0.55	$CaH_4(PO_4)_2 \cdot H_2O$	0.54
$NaH_2PO_4 \cdot 4H_2O$	1.10	Calcium lactate	1.40
RbCl	1.29	Iron lactate	0.12

In the cesium experiments each 5 gm. of the salt mixture contained approximately:

	gm.		*gm.*
$MgSO_4 \cdot 7H_2O$	0.46	$CaH_4(PO_4)_2 \cdot H_2O$	0.45
$NaH_2PO_4 \cdot 4H_2O$	1.27	Calcium lactate	1.12
CsCl	1.59	Iron lactate	0.11

Water was given freely and to it a few drops of iodine solution were added once a week. The animals were put in separate cages, weighed frequently and kept under observation. After varying periods (10 to 15 days) the rats showed marked symptoms of derangement. We noted a sluggishness and general inactivity, followed by a period of marked irritability, with trembling and intermittent twitching which later amounted to tetanic spasms. Within 48 hours after the first tremblings were noted

the rats died in violent tetanic spasms. As a control, the above diet with potassium chloride in amounts equivalent to the rubidium or cesium chlorides was fed to rats with satisfactory results although the same diet plus rubidium or cesium chloride was fatal with the usual symptoms. Moreover a diet in which only one-half of the potassium was replaced by an equivalent amount of cesium or rubidium was lethal. In other words the rubidium and cesium in quantities used were toxic, irrespective of the presence or absence of potassium. The toxicity of rubidium and cesium was more pronounced than we had expected from the literature dealing with a variety of biological experiments in which these salts have been employed. We have found that as little as 2 cc. of 1.44 per cent solution of rubidium

TABLE II.

The Absorption of Rubidium and Cesium by Rat Tissue

Weight of rat at beginning of diet.	Duration of feeding.	Kind of diet.	Weight at end of experiment.	Rubidium or cesium content of tissues weighed fresh.					
				Muscle.	Heart.	Liver.	Kidney.	Spleen.	Lungs.
gm.	*days*		*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
55	17	Rb	50	0.33	0.19	0.22	0.21		
40	17	Rb	39	0.49	0.23	0.34	0.21		
53	13	Cs	57	0.120	0.042		0.060	0.055	0.035
56	10	Cs	46	0.043	0.034	0.039	0.038	0.036	0.017
70	10	Cs	82	0.538	*	0.430	0.555	*	0.219

*Richly present as shown by spectrum. Amount not measured.

chloride (a concentration isotonic with 0.7 per cent sodium chloride), injected into the dorsal lymph sack of frogs weighing from 30 to 40 gm., killed within 24 hours, while 1 cc. on two successive days was also lethal. Autopsy of the rats revealed nothing of note except in one case showing an infection of the lungs. Various tissues of some of the rats which had been on diets containing rubidium or cesium were decomposed in a mixture of sulphuric and nitric acids. The resulting clear solution was made up to 10 cc. and subjected to spectrum analysis for estimation of rubidium or cesium. Results are summarized in Table II. We satisfied ourselves that these metals were actually contained in the cells by a thorough perfusion through a cannula in the left ventricle of a rat, chloroformed after 10 days on the cesium-containing diet. Potassium-free Ringer solution was

employed. The tissues of this animal showed a high content of cesium.

The considerable quantity of rubidium or cesium in the rat muscle, as shown in some of the experiments, is striking. Computed in actual concentration, it amounts in three of the experiments to about half the concentration of potassium as given for normal mammalian muscle (0.32 to 0.42 per cent). This looks like an actual replacement of potassium by rubidium or cesium. We have not yet undertaken the difficult and somewhat uncertain methods for determination of potassium in the presence of such quantities of the interfering substances, rubidium and cesium. The spectroscope, beyond revealing qualitatively that some potassium was present, would not serve the purpose. We cannot, therefore, draw any conclusions as to whether rubidium and cesium are taken into the cell in the place of potassium or in addition to it. It is interesting to note that rubidium and cesium can replace potassium with considerable success in furthering excitability as studied by Zwaardemaker (7) and his coworkers, in antagonistic salt reactions as shown by Loeb (2), and in permitting the development of *Arbacia* eggs as shown by Loeb (8). All of these effects may be due, although proof is not entirely adequate, to action at cell surfaces. In contrast to this, a prolonged action of rubidium and cesium, under circumstances permitting their incorporation into the cell would seem from our observations to constitute physiologically a less successful substitution for potassium.

SUMMARY.

1. Frog muscles perfused with Ringer solution in which potassium chloride has been replaced by an equivalent amount of rubidium or cesium chloride take up rubidium or cesium and incorporate them into the tissue substance in such form as to be retained during a subsequent perfusion with potassium-free Ringer solution, provided the muscles contract during the first perfusion. Retention of rubidium or cesium by a resting muscle does not occur.

2. Rats on synthetic diets, adequate in all respects except that potassium was replaced by an equivalent amount of rubidium or cesium, died after a period varying from 10 to 17 days with characteristic symptoms including tetanic spasms. Muscle, heart, liver,

kidney, spleen, and lung tissues were then found to contain significant amounts of rubidium or cesium. The concentration of these metals in the muscle amounted, in some cases, as shown by a spectroscopic estimation, to about half the concentration of potassium normally found in mammallian muscle.

3. The results are regarded as tending to confirm the theory that the peculiarities in the physiological effects of potassium, including the facility with which it is "selected" by living cells in preference to sodium, are related to the electronic structure of the potassium ion as compared with that of similar ions. The possible relationship of the comparative migration velocity, a function of the electronic structure, to physiological effects is suggested.

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7. Zwaardemaker, H., *J. Physiol.*, 1919–20, liii, 273.
8. Loeb, R. F., *J. Gen. Physiol.*, 1920–21, iii, 229.

THE RELATION OF RESPIRATION TO RHYTHM IN THE CARDIAC GANGLION OF LIMULUS POLYPHEMUS.*

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(Received for publication, September 19, 1921.)

A study of the carbon dioxide production by the cardiac ganglion of *Limulus polyphemus* has shown a definite relation between the rate at which the neurogenic heart beats and the intensity of the respiration of the nerve cells which develop the rhythm. This has been demonstrated by the parallelism between the temperature coefficient of rate of heart beat and of CO_2 production,[1,2] and also by the striking diminution in the CO_2 produced by the nerve cells when the inhibitory nerve to the ganglion is stimulated—a fact which correlates the inhibitory action of the ganglion with the respiratory processes of its nerve cells. These facts at once suggest that automatic impulse formation by the cardiac ganglion of *Limulus*, and probably by all automatic nerve centers, may be determined by the velocity of those chemical reactions in which carbon dioxide is produced by the nerve cells.

The following report presents additional evidence in support of this thesis and shows that a variety of agencies which stimulate the cardiac ganglion and increase the rate of heart beat, produce a similar increase in the rate of CO_2 production by the nerve cells of the heart ganglion.

Method.

The method was the same as that used in previous work.[1,2,3] The color change of phenolsulfonephthalein was used to determine the

* Funds granted by the American Association for the Advancement of Science were used to meet the expenses of this investigation.

[1] Garrey, W. E., *J. Gen. Physiol.*, 1920–21, iii, 41.

[2] Garrey, W. E., *J. Gen. Physiol.*, 1920–21, iii, 49.

[3] Garrey, W. E., *J. Gen. Physiol.*, 1920–21, iii, 163.

rate of change in hydrogen ion concentration which resulted from the formation of CO_2 by the excised cardiac ganglion. The ganglionic cord was immersed in 3 cc. of a standard non-buffer balanced saline solution in small Pyrex glass tubes. The initial pH was 7.8 and the time required to reduce the alkalinity to pH 7.4 was used as an index of the rate of CO_2 formation. To facilitate manipulation the ganglion was draped over glass hooks on a non-soluble glass rod which was cemented into the paraffined cork used to stopper the indicator tube. The standard immersion solution was made by adding 2.2 cc. of M/2 $CaCl_2$ to 100 cc. of M/2 NaCl and the desired initial pH, 7.8, was secured by adding the requisite amount of sodium hydroxide.

EXPERIMENTAL RESULTS.

Electrical Stimulation.—Faradic stimulation of the posterior end of the cardiac ganglion always produces acceleration of the beat of the intact heart. The excised immersed ganglion was similarly stimulated by means of platinum electrodes passed through the cork of the indicator tube. The rate of change in the CO_2 production was compared under this treatment with that of the unstimulated ganglion and the results indicate that during stimulation the rate of CO_2 production is enormously increased, being at least doubled or trebled as shown in Table I. This result is unquestionably due to increase in the chemical processes in the nerve cells, for the faradic shocks do not produce the color changes in the solution, even when the electrodes dip directly into the solution or when applied to a narcotized ganglion. The *decrease* in CO_2 production, when the inhibitory nerve to the ganglion is stimulated, also forms a control experiment which supports the conclusion that we are concerned with a true stimulation of the processes of respiration in the nerve cells in question.

Mechanical Stimulation. Stretching.—A further check upon the results of faradization is obtained by stretching the nerve cord. Carlson[4] had found that distension of the *Limulus* heart cavity increased the rate of the heart beats by its mechanical effect upon the cardiac ganglion. That stretching the nerve cord might produce

[4] Carlson, A. J., *Am. J. Physiol.*, 1907, xviii, 149; *Ergebn. Physiol.*, 1909,viii, 423.

distinct chemical changes seemed likely from analogy with a stretched striated muscle—which increases its osmotic pressure,[5,6] and produces excess of both lactic acid[7] and CO_2.[8]

To determine the rate of CO_2 production by the cardiac ganglion when stretched, silk ligatures were attached to either end of the nerve cord and looped over hooks on the glass mounting rod. By means of these threads it was possible to stretch the ganglion repeatedly, increasing its length some 20 per cent of normal, without apparent injury. The rates of CO_2 production before, during, and after the stretching in five experiments are given as averages in

TABLE I.

Faradic Stimulation and Rate of CO_2 Production.

Condition of ganglion.	Time required to change pH 7.8 to pH 7.4.				
	Experiment No.				
	1	2	3	4	5
	sec.	*sec.*	*sec.*	*sec.*	*sec.*
Normal	192	266	327	840	1620
	221	268	332		
	218		315		
During stimulation	81	130	137	263	255
	100	168			
After stimulation	238	310	367	907	1620
	229	275	342		
	222				

Table II. That the increase during stretching is not merely a result of increasing surface for diffusion, is indicated by the persistent increase in the rate of CO_2 production after the stretching operation had stopped. It is due to the mechanical stimulation of the respiratory rate; and the results thus run parallel to the increase in the rate of heart beat when the ganglion is similarly stretched.

[5] Cooke, E., *J. Physiol.*, 1898, xxiii, 137.
[6] Garrey, W. E., *J. Biol. Chem.*, 1909, vi, p. x.
[7] Gotschlich, E., *Arch. Physiol.*, 1894, lvi, 355.
[8] Eddy, N. B., and Downs, A. W., *Am. J. Physiol.*, 1921, lvi, 188.

Alcohol.—In concentrations of one-half to one per cent by volume the cardiac ganglion of *Limulus* is markedly stimulated by ethyl alcohol[9] and may double the rate of heart beat. These concentrations of purified absolute alcohol, when added to the standard sodium-calcium chloride mixture do not of themselves modify the color of phenolsulfonephthalein used as pH indicator. In these concentrations, however, there results an easily demonstrable increase in the rate of CO_2 production by the ganglion, a result which is again in concord with the interpretation that the increased rate of heart beat depends upon the increased respiration in the nerve cells of the ganglion. Table III records the results of some of the experiments with ethyl alcohol as the stimulating agent.

TABLE II.

Rate of CO_2 Production by Stretched Ganglion.

Condition of ganglion.	Average time to change pH 7.8 to pH 7.4.				
	Experiment No.				
	1	2	3	4	5
	sec.	*sec.*	*sec.*	*sec.*	*sec.*
Normal	200	228	117	251	375
Stretched	142	170	77	163	263
Immediately after stretching	168	200	206	236	318
15 minutes after stretching	190	208		245	335

Sodium Chloride.—In the isotonic concentration, M/2, a sodium chloride solution is a pronounced stimulus to the automaticity of the cardiac ganglion of *Limulus*. The rate of heart beat is promptly accelerated and the individual beats of the muscle merge until a tetanic condition is induced by the continuous discharge of impulses from the ganglionic cells. Determinations of the rate of CO_2 production by the ganglion immersed in M/2 NaCl showed an increase in the rate of respiration of the nerve cells which harmonizes with expectations based on the increase in rate. The rate of CO_2 production was tremendously increased. The averages found in experiments with six ganglia immersed in isotonic NaCl gave the

[9] Carlson, A. J., *Am. J. Physiol.*, 1906, xvii, 177.

following percentages of the normal rate; *i.e.*, 311 per cent, 218 per cent, 237 per cent, 145 per cent, 260 per cent, 134 per cent. Subsequent to these determinations immersion of the ganglia in the sodium-calcium solution demonstrated the antagonism of these ions in their effects on animal oxidations for the mixture caused a return of the rate of respiration to within 15 per cent of the previous normal in every instance. This fact excludes the possibility that the increase in CO_2 formation in the isotonic NaCl is a lethal phenomenon, in the ordinary sense of that term, or at least that the stages dealt with

TABLE III.

Effects of 1 per cent Ethyl Alcohol on Rate of CO_2 Production.

Condition of ganglion.	Time required to change pH 7.8 to pH 7.4.					Average rate of CO_2 production.
	Experiment No.				Average time of all experiments.	
	1	2	3	4		
	sec.	*sec.*	*sec.*	*sec.*	*sec.*	*per cent*
In Na/Ca solution (normal).....	184	265	315	448	305	100
In Na/Ca + 1 per cent ethyl alcohol.	110	150	145	245	160	190
	90	140	150	265		
	102		165	290		
In Na/Ca solution.....	130	148	230	485	296	103
	147		255	230		
	201	248				
In Na/Ca solution + 1 per cent alcohol	152	166	160	315	198	154

are still reversible (Osterhout)[10]. Loeb and Wasteneys[11] working with concentration of sodium chloride isotonic with sea water found a decrease in oxidation in eggs of *Arbacia* and of *Gonionemus*; this apparent disharmony with the behavior of *Limulus* heart ganglia may be specific for the material used or may be merely an expression of the duration of exposure to NaCl or of the concentrations of the sodium chloride to which the tissue is subjected since Brooks[12] has

[10] Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21, iii, 15.

[11] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1910, xxviii, 340; 1911, xxxi, 168.

[12] Brooks, M. M., *J. Gen. Physiol.*, 1919–20, ii, 5.

shown that one can get an increase or a decrease in the respiration of *Bacillus subtilis* depending upon the concentration of NaCl employed.

Adrenalin Chloride.—Addition of one drop of adrenalin chloride solution (1 part to 10,000 parts of solvent) to each cubic centimeter of the standard sodium-calcium solution stimulates the ganglion and increases the heart rate[9]. Experiments with three ganglia immersed in this concentration of the drug gave the average rate of CO_2 production as 204 per cent, 194 per cent, and 166 per cent of the normal.

These experiments amply justify the generalization that those stimuli, which acting on the cardiac ganglion to cause an increase in the rate of the heart beat, likewise produce changes which result in a marked increase in the respiratory processes within the nerve cells. Impulse formation by the ganglion, and CO_2 production are therefore intimately related processes and appear to stand in relation of effect and cause respectively. A complete justification of this postulate requires that we shall be able to establish a quantitative correspondence between the processes of impulse formation, that is rate of heart beat, and the rate of CO_2 formation. Experiments directed to this end could not be conducted in such a way as to record both the rate of heart beat and the rate of CO_2 production on the same preparation simultaneously, but it was possible to secure a satisfactory approximation by taking advantage of the characteristic effects produced by changes of temperature especially the effects of transient exposure to relatively high and low temperatures. These are considered in the following paragraphs.

Effects of Temperature Extremes (Transitory Exposures).

Reference has been made in previous communications[1,2] to the fact that if the ganglion is exposed for a few minutes to a very low temperature (0°C.) and then warmed to 10°C., for example, a new rate of rhythm is established which is faster than was obtained at this temperature before cooling. This increase in rate may amount to 25 per cent or 30 per cent. A similar effect upon the rate of carbon dioxide produced by the ganglion has been noted under like treatment, and eight examples have been referred to in Tables I and II of a former paper.[2] In four experiments more recently carried out the times required for the color change in our indicator at 10°C.

were 408, 532, 618, and 803 seconds, but after exposing the ganglia for five minutes to 0°C. then again warming to 10°C. the time was in each case shortened being respectively 320, 416, 482, and 629 seconds. The increase in the rate of CO_2 production averages 28 per cent in these experiments and is in good quantitative correspondence to the acceleration rate of rhythm as stated above.

When on the other hand ganglia are exposed to the upper extremes of temperature compatible with function, *e.g.*, to 35°C. or 40°C., and are subsequently cooled the rate of rhythm is much *slower* at the lower temperature than before warming process; thus in five experiments in which the rates at 25°C. were determined before and after heating the ratios of the actual rates of beat were 20:17, 20:13, 18:13, 24:20, and 17:8. This corresponds to an average decrease to 70 per cent of the previous rate. Four other experiments in which similar conditions obtained indicated a depression in the rate of CO_2 to a point only 78 per cent of the value before exposure to the high temperature. These values for both extremes of this temperature range again show a very good quantitative correspondence between the effects upon rate of heart beat and rate of CO_2 formation by the ganglia.

The quantitative relationship between the two processes is made much more striking by the graph given in Fig. 1. This gives the curve for a single typical experiment in which the rates of beat are plotted as ordinates against temperature as abscissæ. In this experiment the ganglion was progressively cooled from room temperature to 0°, then warmed to 38°, and again cooled. The direction of the temperature change is indicated by the arrows and the rate by outline characters, triangle, circle, and square, respectively.

A companion graph was constructed for the rate of CO_2 formation. For this purpose eleven experiments were taken in which the ganglia had been treated to the same progressive changes in temperature as in the previous experiment on rate of heart beat. The average time required for CO_2 formation was determined for different temperatures. It was found that if the rate of CO_2 formation was expressed as the reciprocal of the time in seconds and this was multiplied in each case by the constant 1400 a plot of the results gave a curve identical with that shown in Fig. 1. The points obtained by this calculation

have been introduced into Fig. 1 as solid characters. This striking quantitative parallelism between rate of heart beat and rate of development of CO_2 in the cardiac ganglion, which is brought out by this graph, forcefully substantiates the probability that the two

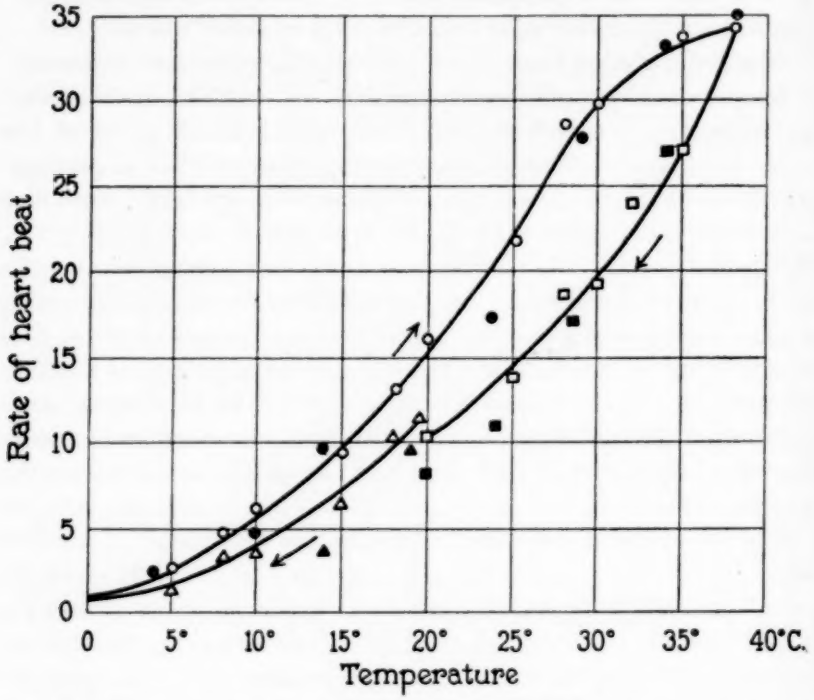

FIG. 1. Curve of rate of heart beat when the ganglion is initially cooled from the normal temperature to 0°C. (△), then progressively warmed to 38°C. (○), and finally cooled to room temperature (□). The solid characters indicate the corresponding average rates of carbon dioxide production by the ganglion.

physiological processes are bound together in the relationship of cause and effect and that it is the velocity of those chemical reactions which give rise to carbon dioxide formation in the cells of the cardiac ganglion which determines the rate of the neurogenic heart of *Limulus polyphemus*.

COMPARATIVE STUDIES ON RESPIRATION.

XIX. A Preliminary Stage in the Progress of Ether Anesthesia.

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(Received for publication, September 1, 1921.)

One of the fundamental aspects of the action of the anesthetics is their effect upon respiration. In order to study this under conditions where the anesthetic is the only variable, it is desirable to employ plants, since experiments with animals are usually complicated by muscular action and its influence on the rate of respiration. Under suitable conditions the rate of respiration in plants remains remarkably constant for periods of time considerably longer than the average experiment, so that they offer good material for this type of work.

The method employed in the writer's experiments was that described by Osterhout.[1] The material used was wheat, which was chosen because it is easy to grow, and gives very constant results. The wheat was a pure strain, supplied by the Minnesota Experimental Station, and was particularly well suited to the purpose, being uniform in growth and showing a high viability. The seeds were germinated with aseptic precautions, being grown in sterile paper cups in a saturated atmosphere in the dark. The greatest care was necessary to avoid contamination by molds, and the most effective way of checking them was found to be that of soaking the dry seeds for at least 10 minutes in full strength commercial hydrogen peroxide before germinating. Cultures showing the slightest sign of molds were rejected, as they gave aberrant results. It was found that by allowing no liquid water in the cultures after the roots had begun to emerge, but by keeping the atmosphere saturated (by means of a bell jar inverted over the Petri dish full of water in which the paper cups stood), the seeds produced an abundance of very well-developed

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17.

root-hairs. The material was used when the roots were from one to one and a half inches long, with abundant surface for absorption and respiration. Before using, the seeds were washed for 10 minutes in running water. They were then put into a flask of Pyrex glass, with 100 cc. of distilled water, and the apparatus was allowed to run. After the rate of respiration had become constant (in distilled water) it usually remained so for at least 8 hours. As the average experiment lasted from 2 to 4 hours, it was possible to discount any variations in the rate as being due to manipulation, provided that the temperature remained constant. During the course of an experiment the temperature seldom varied more than a degree either way. The average room temperature was 20°C.

The indicator used was phenolsulfonephthalein in aqueous solution. The normal rate of respiration was taken as the reciprocal of the time required to change the pH value of the indicator from pH 7.36 to pH. 7.09, these values being chosen as being sufficiently different in tint to be easily read. The time varied with the age of the seedlings, ranging between 30 and 60 seconds.

The seeds were treated with a watery solution of ether, the concentrations employed being 1 per cent, 3.65 per cent, and 7.3 per cent (by volume). After the rate of respiration had become constant, 100 cc. of the required solution was substituted for the distilled water in the flask, and the experiment continued.[2]

With a solution of 1 per cent ether in distilled water the first effect was a fall in the rate of respiration. Fig. 1 (average of five experiments) shows that the minimum reached was 36 per cent of the normal, 6 minutes after the beginning of exposure to the ether. The rate then rose gradually towards normal, but remained below it for 48 minutes. The next stage was a rapid rise to 124 per cent, the maximum being reached in 51 minutes, and the duration above normal being 6 minutes. This was succeeded by a depression, when the rate fell gradually to about 50 per cent in about 4 hours.

With a solution of ether 3.65 per cent the first effect was the same, the rate reaching a minimum of 48 per cent in 9 minutes. The depression lasted for 24 minutes, and was followed by a rapid rise, the

[2] Care was taken to avoid any possible errors due to the admission of air in opening the apparatus or to the introduction of distilled water.

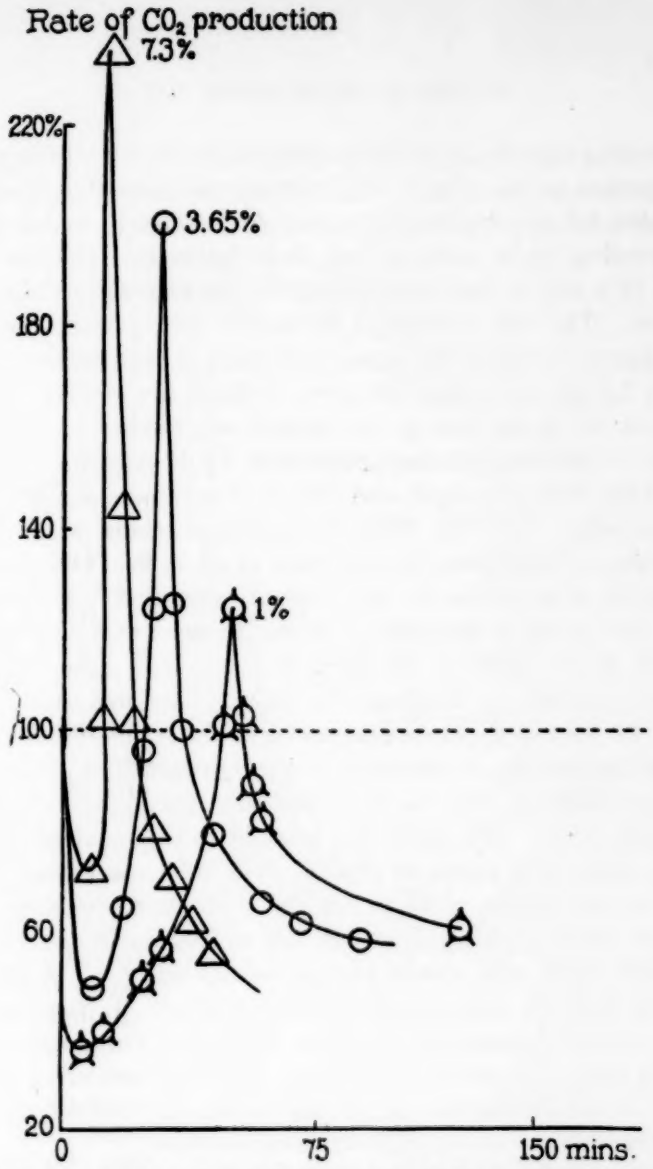

FIG. 1. Curves showing the rate of respiration of wheat seedlings (expressed as per cent of the normal), in 1 per cent, 3.65 per cent, and 7.3 per cent ether (by volume). The normal rate (which is taken as 100 per cent), represents a change in pH value from 7.36 to 7.09 in a number of seconds varying with the age of the material, usually between 30 and 60 seconds. The curve with 1 per cent ether represents the mean of five typical experiments (probable error of the mean, less than 10 per cent of the mean). The curve with 3.65 per cent ether represents the mean of ten typical experiments (probable error of the mean, less than 5 per cent of the mean). The curve with 7.3 per cent ether represents the mean of six experiments (probable error of the mean, less than 5 per cent of the mean, except for two points, where the error is 10 per cent of the mean).

rate reaching a maximum of 200 per cent in 30 minutes after beginning the exposure to the ether. This second rise undoubtedly masked the earlier fall in experiments by the older methods, which did not allow readings to be made at such short intervals. The rise is succeeded by a fall, so that after 78 minutes the rate was reduced to 60 per cent. The rate continued to decline slowly after this. The above figures represent the mean of ten typical experiments.

With 7.3 per cent ether, the general effects are similar. A minimum rate of 70 per cent of the normal was reached in 9 minutes, and the respiration remained subnormal for 12 minutes. The succeeding rise was very rapid, and reached a maximum of 235 per cent in 15 minutes. The rate then fell rapidly, reaching 60 per cent in 39 minutes. These data are the mean of six typical experiments.

In order to determine if the observed preliminary fall was really due to the action of the ether, and not to some experimental error inherent in the method, the work was repeated, using the direct indicator method as developed by Haas. The seeds were placed in a Pyrex tube of the same dimensions as the standard buffer tubes used for comparison, and the normal rate determined by adding measured quantities of tap water, containing phenolsulfonephthalein, 5 drops to 10 cc. The water was brought to the required pH value by the addition of traces of NaOH. The tube was closed, and inverted several times, so as to mix the contents thoroughly. After the time taken to change from pH 7.36 to 7.09 was noted, the seeds were well rinsed with several changes of tap water. The ether was made up with tap water and the indicator added. It was found that the results were essentially the same. That is, a curve was obtained showing three well marked divisions: a preliminary fall, a rise, and a final slower declension. It is thus reasonably certain that these results are due to some specific action of the ether.

The increase in rate followed by a fall was also observed by Thomas[3] in experiments on wheat but she does not describe a preliminary depression. In regard to the latter the results recall those of Irwin[4] on frog eggs and *Fundulus* embryos. In these cases Irwin found that the first effect of ether, in certain concentrations, was a fall in the rate

[3] Thomas, H. S., *J. Gen. Physiol.* 1918–19, i, 203.

[4] Irwin, M., *J. Gen. Physiol.* 1918–19, i, 209.

of respiration. The significance of these results seemed to be doubtful, and no emphasis was laid on them. The present experiments on plants, therefore, indicate that this preliminary depression may be

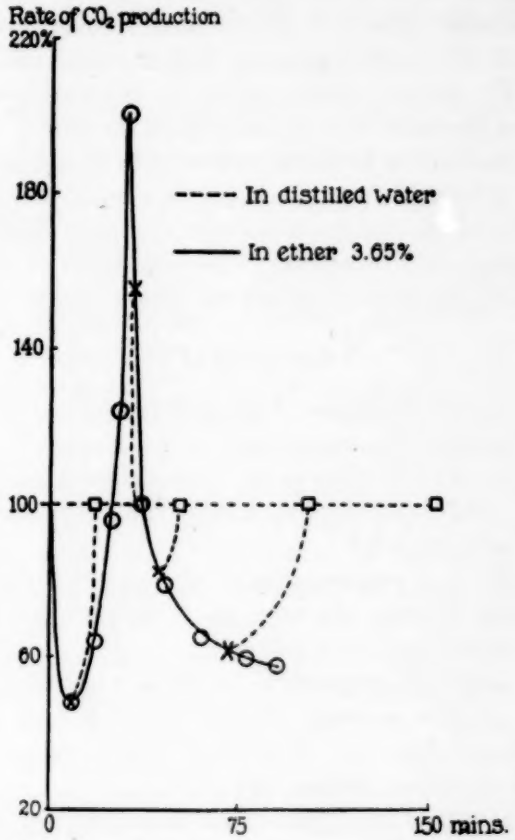

FIG. 2. Curves showing recovery from exposure to ether. Normal rate as in Fig. 1. The solid line represents respiration in ether; the broken line, respiration in distilled water. The points marked × on the curve indicate points of removal from ether. The curve represents the mean of six experiments (probable error of the mean, less than 5 per cent of the mean).

of general occurrence. In that case their importance is evident, and must be reckoned with in any attempt at a general theory of anesthesia.

The interpretation of these results raises several questions. For example, which part of the curve represents stimulation, which anesthesia, and at what point do the toxic (irreversible) changes begin? In regard to the latter, it was found that, even when the respiration had been reduced below normal, complete recovery was possible on removal to distilled water, provided that the depression was not too great (Fig. 2). Below a certain range the respiration fell even more rapidly when the seeds were removed from the ether. There was no evidence of incomplete or partial recovery.[5] It is thus seen that up to a certain point, which may be below the normal rate of respiration, the curve represents changes in the rate of carbon dioxide production which are completely reversible. The boundaries of the stimulatory and anesthetic effects must be left for future experiments to decide.

SUMMARY.

Using these concentrations of ether (1 per cent, 3.65 per cent, 7.3 per cent), the following conclusions may be drawn.

1. The first effect of ether is to cause a depression in the rate of respiration. This is followed by a rapid rise above normal, which in turn is succeeded by a fall.

2. With all these concentrations the respiration is ultimately reduced to approximately the same level; the stronger the ether, the less time required to produce this result.

3. Even when the respiration has been reduced below normal, recovery is possible on removal from the ether, and appears to be complete, if sufficient time is allowed. If, however, the rate has been too far depressed, no recovery is possible.

4. These results extend those of Irwin on frog eggs and *Fundulus* embryos.

[5] These experiments were made by Osterhout's method.[1]

STEREOTROPIC ORIENTATION OF THE TUBE FEET OF STARFISH (ASTERIAS) AND ITS INHIBITION BY LIGHT.*

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(Received for publication, Sept. 19, 1921.)

Recently Maxwell has shown that the reactions of sharks to contact stimuli are due to changes in the relative tension of the antagonist muscles similar to those taking place in the galvanotropic, heliotropic, and geotropic reactions of animals.[1] The writer has recently made observations on the starfish which show that contact stimuli applied to the sides of a ray bring about changes in the orientation of the tube feet which are comparable to heliotropic reactions. These stereotropic reactions of the starfish gain especial significance since they can be inhibited by the reaction to light.

In order to demonstrate the stereotropic orientation of the tube feet, the starfish is laid on its back in a dish of sea water. If the animal is prevented from righting itself for 1 or 2 minutes it becomes comparatively quiet; if now a contact stimulus is applied to one of the rays by pressing a foreign body such as a piece of cork, a glass rod, or a finger tip against the side of the ray, a retraction of the tube feet and closure of the ambulacral groove occurs. Next, the groove opens and the tube feet move toward the stimulated area. The reaction is especially marked in the immediate vicinity of excitation but in the more sensitive individuals it involves the entire ray. The average length of time which elapses between the moment of contact excitation and the protrusion of the tube feet is 2.8 seconds. The contact stimulus may be applied momentarily and removed before the reaction begins, but the series of reactions proceeds as

* The experiments described in this paper were done in the Botany Laboratory at Woods Hole, and the writer wishes to express his thanks to Professor Osterhout for many courtesies extended to him during the progress of the work.

[1] Maxwell, S. S., *J. Gen. Physiol.*, 1920–1921, iv, 19. von Uexküll, J., *Z. Biol.*, 1900, xxxix, 73.

described. This gives confirmatory proof of the machine-like character of the reaction. It is also worthy of note that the circumoral nerve ring plays no part in the reaction, but only the radial nerve through which the tube feet receive their impulses since these experiments can be made on isolated rays. Fig. 1 is a diagram showing the orientation of the tube feet which are extended toward the point of contact stimulation.

If two points on the same side of the ray but at a distance from each other are touched, then the tube feet turn to that side (Fig. 2). The tube feet midway between the two loci of stimulation bend neither toward the one nor toward the other but at right angles to a line

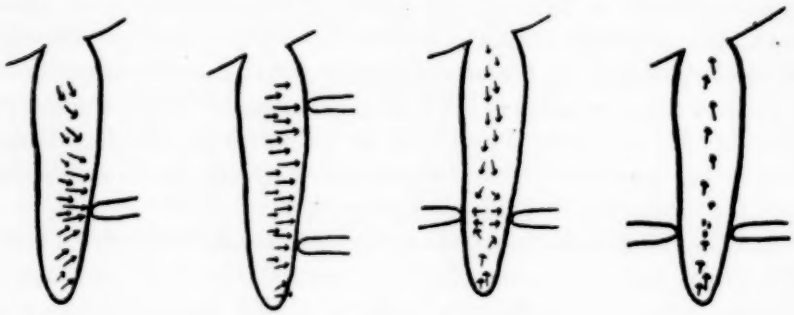

FIG. 1. FIG. 2. FIG. 3. FIG. 4.

FIG. 1. Arrows indicate the direction in which the tube feet are bent, the arrow tip being put for the terminal disks. This diagram shows the tube feet all inclined accurately to the locus of contact excitation indicated by the U-shaped outline.

FIG. 2. Two loci of contact excitation on the same side of the ray result in the tube feet orienting themselves at right angles to the axis of the ray and to a line joining the two loci of stimulation. The median tube feet do not incline to either point of excitation but orient like a phototropic insect placed midway between two lights of equal intensity.

FIG. 3. Slight contact excitation has been applied at two points on opposite sides of the ray. Only the tube feet in the immediate vicinity are directed toward the points of excitation. The other tube feet are directed along the median axis of the ray at right angles to a line joining the two loci of stimulation.

FIG. 4. The same as in Fig. 3, except that the pressure is strongly applied resulting in withdrawal of the tube feet in the immediate vicinity of excitation, and orientation of the others to the center parallel with the axis of the ray and perpendicular to a line joining the two loci of excitation.

joining the two points. In this we have a tropistic reaction analogous to that of heliotropic orientation to two sources of light.[2] The same principle may be illustrated in another way by gently pressing the ray between two small bodies such as glass rods. When the extension reflex takes place it will be seen that only a few of the tube feet bend laterally and they are in the immediate vicinity of the points touched. All the other tube feet bend along the axis of the ray toward the area of excitation, swaying a little from side to side, but neither markedly to the right nor to the left (Fig. 3). If the pressure is increased the tube feet central to the point of stimulation reverse their orientation and bend toward the center (Fig. 4). However, not every animal gave both phases of this reaction; *i.e.*, distal and central bending of the tube feet. In the main the results were similar to those obtained by Maxwell with *Mustelus* in which he found that weak mechanical stimulation caused bending toward the point of contact while strong stimulation produced the opposite result.[1] It happens therefore that when acted upon by contact on two opposite sides of the ray, *i.e.*, by two equally balanced impulses, the tube feet orient themselves along a line perpendicular to a line joining the two loci stimulated. Here again is a case analogous to that of heliotropic orientation to two sources of light, since the starfish ray like the heliotropic insect is bilaterally symmetrical with reference to right and left.

If the tube feet as a result of their extension in response to contact touch a surface, they at once adhere by means of their sucking disks. When a considerable number of tube feet have thus taken hold it is difficult to pull the animal away from a surface. Even if one succeeds in doing so some of the tube feet will be torn from the animal and left sticking to the surface, so strong is the hold they have upon it. It is, however, possible by means of the light reaction to cause adhering starfish to release their hold. This reaction may be demonstrated as follows: A starfish is placed ventral side up in a dish of sea water in a dimly lighted room. As soon as the tube feet have been thrust out, a flash of sunlight is thrown across the animal. As a result the tube feet withdraw and the ambulacral

[2] Loeb, J., Forced movements, tropisms, and animal conduct, Philadelphia and London, 1918, 75. Patten, B. M., *J. Exp. Zool.*, 1914, xvii, 213.

grooves close; the rays bend ventrally. After several seconds in this position the grooves open and the tube feet are extended. This occurs even if the illumination is continuous.

For purposes of better control all the experiments with light were made in the dark room. While the retraction is uniformily elicited in the dark-adapted starfish with white light of sufficient quantity; red light has no such effect. It was therefore practicable to observe the animals at any time by means of red light while white light was admitted by a shutter for any desired length of time. Use was made of the lamp and optical bench described by Hecht.[3] The source of white light was a 260 candle-power Mazda lamp. The time of exposure was measured with a stop-watch. The exposures were also checked by means of the shutter of a photographic camera. Each starfish was kept in a rectangular glass dish during a series of experiments. The test was made by allowing the light to fall perpendicularly on the side of the dish on which the animal rested. This procedure resulted in illuminating the ventral sides of one or more rays. Withdrawal of the tube feet and beginning closure of the groove were taken as the end-point of the reaction. In case it was desired to avoid contact on the part of the tube feet, the animal had to be supported vertically in the dish while the exposure was made. The starfish must be kept in the dark for an hour before beginning the experiments and they must not be excited mechanically at the time of the test. It was found that a subliminal exposure to light preceding by a few seconds an otherwise adequate exposure, completely inhibited the reflex. Therefore only one measurement could be made at a time. Accordingly after each exposure the animals were put into freshly aerated sea water and kept in the dark for 15 minutes before being tested again.

The shortest reaction time obtainable with a light intensity of 26,000 candle-meters intensity was 1.5 seconds. The longest reaction time secured with a weak light was approximately 3 seconds. If the light intensity was so low that an exposure of more than this length of time was necessary to produce the required photochemical effect, no reaction was obtained. The minimum quantity of light

[3] Hecht, S., *J. Gen. Physiol.*, 1919–1920, ii, 229.

which would bring about the reaction in a dark-adapted starfish, the tube feet not being in contact with a surface, was found to have an average value of 10 to 25 candle-meter seconds.

Illumination of the dorsal surface of the starfish does not cause the retraction of the tube feet nor closure of the ambulacral groove. This shows either that the dorsal surface is insensitive to light or that nervous connection between the sensory cells of the dorsal surface and the tube foot musculature is lacking. Since, as we know from the effects of mechanical stimulation[4] there is nervous connection between the stereosensitive cells of the dorsal surface and the tube feet, the first hypothesis is probably correct; *i.e.*, the dorsal surface has no light receptor cells.

The "feelers" of the tips of the rays are relatively insensitive to light since they show no retraction upon illumination. Only tube feet with well developed terminal pads are strongly photosensitive. Illumination of a limited number of tube feet causes reaction only in that area, or at most, in the most sensitive individuals, only in the ray illuminated. The light reaction is therefore local in character.

It was noted in the experiments that tube feet which were not in contact with a surface retracted much more readily in response to illumination than did those which were in contact with a surface. But by the use of more intense light it was found possible to force the retraction of those in contact with a surface. This antagonism between stereotropism and the reaction due to light gives a means of quantitative treatment of stereotropism by the method of indirect measurement. It is therefore only necessary to illuminate the animal with a known quantity of light just sufficient to neutralize its stereotropism, as shown by the withdrawal of the tube feet from the surface, in order to have a measure of stereotropism in terms of light quantity. Although 10 to 25 candle-meter seconds is sufficient to cause retraction of tube feet which are simply extended in the water without touching a surface, this quantity of light has no apparent effect upon the tube feet which are in contact with the glass side of the aquarium. But if exposed to a light sufficiently powerful, starfish clinging to the side of a glass dish frequently release their hold en-

[4] Moore, A. R., *J. Gen. Physiol.*, 1919–20, ii, 319.

tirely and drop to the bottom. The least quantity of light which will cause the retraction of the tube feet from a surface may be regarded as the photic equivalent of stereotropism.

In making determinations of the photic equivalent of stereotropism the apparatus and procedure were the same as noted above except that the exposures were made on the ventral side of the starfish while it clung to the vertical wall of the dish. It was thus possible to measure the distance of the receptors from the light source with accuracy. Exposures were made on each animal at 5 cm. intervals (Table I). The letters in the table indicate the individual animals. The figures under the letters are the distances in centimeters at which the 260 candle-power light acting for the time interval stated in the first column will just cause the tube feet to be withdrawn from the wall and the ambulacral groove to begin to close. At a distance

TABLE I.

Exposure time.	A	B	C	D	Average i.	$t \times i$*
secs.						
0.5	70	60	75	60	609	305
1	85	90	110	90	304	304
2	125	130	160	140	139	278

* i represents intensity in candle meters; t, exposure time in seconds.

of 5 cm. farther away from the light an exposure of the length designated caused no significant response. Each figure is the result of repeated trials.

While there is a considerable variation in the values when different individuals are compared, each animal yields fairly consistent results. For example, *C* has higher sensitivity than the others and shows the fact in each of the three exposures. The desirability of more extensive studies is apparent, but the end of the season cut short the progress of the experiments and the data given are submitted for the purpose of illustrating the possibilities of the method.

As a result of the measurements made, it can be stated that the average photic equivalent for stereotropism in *Asterias* is between 250 and 350 candle-meter seconds. This is a value 10 to 20 times as great as is required to cause retraction of the tube feet when they are

not in contact with a surface. It is also clear from the figures in the table that the length of exposure is inversely proportional to the light intensity since the product of intensity by time equals a constant. This shows that a certain quantity of light is required to bring about the reaction, which is but another way of saying that the Bunsen-Roscoe law holds here as it does in other photochemical reactions.[3,5]

[5]Loeb, J., Forced movements, tropisms, and animal conduct, Philadelphia and London, 1918, 83.

COMPARATIVE STUDIES ON RESPIRATION

XX. The Cause of Partial Recovery.

By O. L. INMAN.

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(Received for publication, September 29, 1921.)

New questions regarding the mechanism of injury and recovery have recently arisen. It has become apparent that, contrary to the accepted view, recovery from injury may be either partial or complete, according to circumstances. This conclusion was drawn from investigations on electrical conductivity by Osterhout[1] and has been confirmed by the experiments on respiration carried out by the writer.[2]

A question of especial interest is whether partial recovery is due to the death of certain cells or to a lowered rate of metabolism of all the cells which make up the tissue. The writer has endeavored to throw light on this question by determining the number of cells that survive after exposure to a toxic agent and by comparing the rate of respiration before and after such exposure.

The material used in these experiments was a unicellular alga, *Chlorella*. It was isolated from the soil by Dr. Schramm and grown on agar, free from bacteria and other organisms. The agar was prepared in the following manner.

Agar	10 gm.
Calcium nitrate	1.026 gm.
Magnesium sulfate (hydrated)	0.2 gm.
Dipotassium phosphate	0.2 gm.
Calcium chloride (anhydrous)	0.1 gm.
Ferrous sulfate	trace
Distilled water	1000 cc.

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21, iii, 145, 415, 611.

[2] Inman, O. L., *J. Gen. Physiol.*, 1920–21, iii, 663.

These salts were dissolved in water; the agar was then added and the whole mixture sterilized in an autoclave.

From agar the alga was transferred to a liquid medium prepared in the same manner with the exception that 10 gm. of dextrose were used in place of 10 gm. of agar.

The cultures used in these experiments were grown for a period of 30 days in this liquid medium. The medium has a very slight alkaline reaction and when tested showed no detectable buffer action.

The algæ were transferred to the respiration chamber[3] along with enough of the medium to make a volume of about 20 cc. The normal rate of respiration was determined by taking the time necessary for a change from pH 7.78 to pH 7.36. (This usually took about 2 minutes). When the normal rate of respiration was practically constant the algæ were separated from the medium by means of a centrifuge, which was run at a moderate speed. (Tests showed that this centrifugation caused no injury.) The reagent was then added and measurements of the rate of respiration were made at frequent intervals until the desired point below the normal rate of respiration was reached; the algæ were then returned to the normal solution and at intervals the rate of respiration was measured.

The experiments were performed at 18 ± 3°C.

Fig. 1 shows the result of exposing the cells to hypertonic balanced solutions. If the respiration does not fall below 60 per cent of the normal, recovery is complete; if it falls lower recovery may be incomplete, while if the rate drops too low there is no recovery.

It was found in these experiments that there is no tendency for recovery to complete itself even when the organism is kept for days under the most favorable conditions.

Fig. 2 shows recovery after exposure to solutions of chloroform. In this, as in the previous case, no attempt was made to follow the respiration curve during the exposure since the interesting question was the amount of injury produced and the subsequent behavior. Curves *A*, *B*, and *C* show that if the injury is stopped when respiration is from 58 to 70 per cent of normal, there is in every case complete recovery within a period of about 4 hours and that the organism

[3] This is the apparatus described by Osterhout. Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17.

is found at the end of 20 hours to be still normal. Where complete recovery was observed the algæ were planted upon agar plates and they grew and formed colonies in the normal manner.

Curves *G* and *F* show that there is no partial recovery but a continued state of diminished metabolism which lasts over a considerable number of hours as shown by the curves.

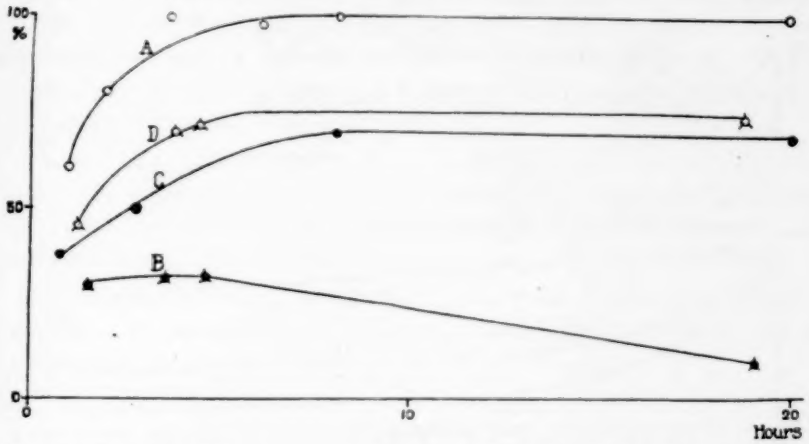

FIG. 1. Curves showing recovery of *Chlorella* from the effects of a hypertonic balanced solution of NaCl plus $CaCl_2$. Curve *A* shows the degree of recovery after an exposure of 60 minutes to a solution containing 50 cc. NaCl 1 M to each cc. of $CaCl_2$ 1 M; Curve *D*, after an exposure of 78 minutes to the same solution; Curve *C*, after an exposure of 48 minutes to a solution containing 50 cc. NaCl 1.25 M to each cc. of $CaCl_2$ 1.25 M; Curve *B*, after an exposure of 96 minutes to the last mentioned solution. The first point on each curve denotes the per cent to which the respiration had fallen when the algæ were transferred from the solution to the normal medium. The ordinates represent the rate of production of CO_2 expressed as per cent of the normal; the abscissæ, time in hours. Each curve represents a typical experiment.

Curves *D* and *E* show that when the respiration has fallen to about 38 per cent of the normal, as the result of the exposure to chloroform, there is no recovery after removal from the chloroform, but, on the contrary, a gradual decrease in the rate.

Experiments were next undertaken to decide whether partial recovery meant that some of the cells had been killed and others had remained uninjured, or whether all the cells remained alive but had

a lowered rate of metabolism because of the treatment. In order to throw light upon this point it was decided to resort to staining methods. Methylene blue was found to be convenient since it would quickly stain the algæ that had been killed by the chloroform or the hypertonic salt solutions, but did not stain the living cells during a short exposure to the dye. In the normal culture some of the cells (from 5 to 8 per cent) were stained.

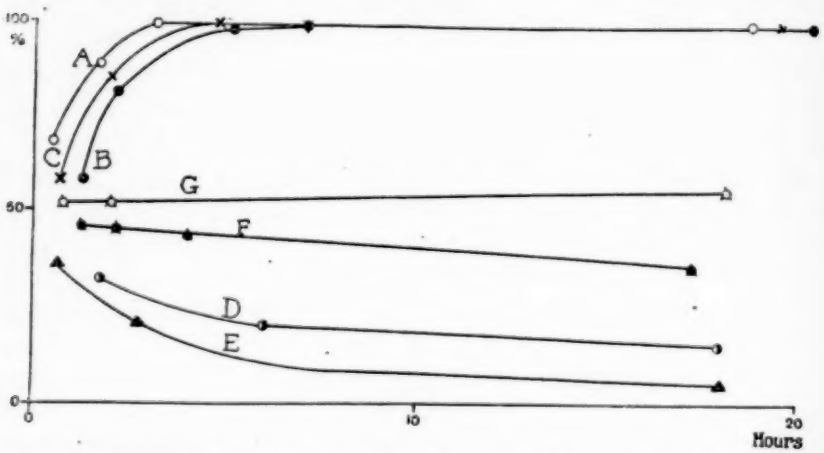

FIG. 2. Curves showing the rate of respiration of *Chlorella* after treatment with chloroform. Curves *A*, *B*, and *C* were obtained by using 0.225 per cent chloroform (by volume); Curves *G* and *F* with 0.27 per cent; Curve *B*, with 0.36 per cent. The first point on each curve denotes the per cent to which the respiration had fallen when the algæ were transferred from the solution of chloroform to the normal medium. The ordinates denote the rate of production of CO_2 expressed as per cent of the normal; the abscissæ, time in hours. Each curve represents a typical experiment.

Cultures of the same age were selected and the normal respiration was measured in the usual manner. Each culture was then treated with chloroform of sufficient strength to decrease the respiration of the algæ. The algæ were then subjected to the stain (for 3 minutes) and it was found that not more than 8 per cent of the cells were ever stained,[4] no matter whether the respiration had fallen to 70 per cent or to 10 per cent of the normal rate. The time of exposure of the

[4] The cells were counted by means of a hemocytometer.

algæ to the chloroform was never more than 96 minutes. After an excessively long exposure to the chloroform (10 to 15 hours) about 95 per cent of the cells stained; in this case there is little doubt that most of the cells were killed, but such long exposures were never used in the ordinary experiments.

Staining was also tried after treatment with hypertonic salt solutions. Here partial recovery was found and yet not more than 5 to 8 per cent of the cells stained. In these experiments the longest time of exposure to the salt solution was about an hour. If the cells were treated for from 5 to 8 days with hypertonic NaCl about 95 per cent stained.

It would therefore appear that a treatment with hypertonic salt solutions which lowers the rate of respiration so much that recovery is incomplete kills few or none of the cells. If this be true then it cannot be said that partial recovery is due to the fact that some of the cells are killed by the toxic agents while others recover completely. The fact that the recovery is not complete must be due to the diminished metabolism of practically all the cells.

SUMMARY.

The respiration of *Chlorella* is diminished by exposure to hypertonic salt solutions. After a short exposure there is complete recovery when the algæ are removed to the normal medium. After a longer exposure recovery may be incomplete, as shown by the fact that the rate of respiration fails to rise to the normal level. Staining with methylene blue indicates that few, if any, of the cells are killed as the result of the exposure. It would therefore seem that the treatment produces a persistent lowering of the rate of metabolism. Such a condition of metabolism is also found after exposure to chloroform.

THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE PRODUCTION OF CARBON DIOXIDE BY BACILLUS BUTYRICUS AND BACILLUS SUBTILIS.

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(Received for publication, October 3, 1921.)

In connection with the study of various therapeutic agents and their effects upon the carbon dioxide production of bacteria, it was necessary to investigate the influence of changes in the hydrogen ion concentration before any conclusions could be drawn.

Clark[1] has given a comprehensive bibliography of the influence of hydrogen ion concentration upon various activities. Clark and Lubs[2] have given a summary of the limiting reactions which govern the growth of some bacteria. Most of the literature, however, is concerned with metabolic activities other than the production of carbon dioxide. Thunberg[3] has observed the effects of various hydrogen ion concentrations upon surviving frog muscle, and has found that there was a considerable decrease in the production of CO_2 in both the acid and the alkaline range.

Gustafson,[4] using substantially the same method as the writer, and working with *Penicillium chrysogenum*, obtained a decrease in the rate of CO_2 production when acid was added in quantity sufficient to make the pH 4 or less. He also found a decrease in the rate when the pH of the medium containing the organisms was 8 or more. Between these two concentrations he found no variation in the rate of CO_2 production.

The method used by the writer was that described in a previous publication.[5] The apparatus is a closed system, containing at either

[1] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.

[2] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1, 222.

[3] Thunberg, T., *Skand. Arch. Physiol.*, 1910, xxiii, 154; 1911, xxiv, 23.

[4] Gustafson, F. G., *J. Gen. Physiol.*, 1919–20, ii, 617.

[5] Brooks, M. M., *J. Gen. Physiol.*, 1919–20, ii, 5.

end two Pyrex glass tubes, in which are placed respectively the bacteria to be used and the indicator, the change in color of which measures the respired CO_2 which is forced through the system.

In order to facilitate experimentation, a non-pathogenic acid-fast organism was used. This was *Bacillus butyricus*, obtained from the stock culture of the Hygienic Laboratory, and isolated originally from butter. The rapid growth of this organism is an advantage; 48 hour cultures grown on glycerin agar and incubated at 37°C. were used for all the experiments. The heavy growth obtained was washed off into a 0.75 per cent solution of dextrose in distilled water.

As a basis of comparison, the same experiments were also performed with *Bacillus subtilis*, a non-acid-fast organism, which had been planted upon agar-agar and incubated for 18 hours at 37°C. previous to use. These organisms were then transferred to a 0.75 per cent solution of dextrose in distilled water.

For each experiment 2.5 cc. of 0.75 per cent dextrose solution containing the organisms were used. The dextrose solution had a pH of 7.0. The hydrogen ion concentration of the solution was changed by adding drops of various concentrations of NaOH or H_2SO_4 from a standard dropper. In solutions requiring a greater amount of acid or alkali more concentrated solutions were used, thus keeping the volume nearly constant.

The NaOH was prepared from the best reagent obtainable, handled with all necessary precautions to avoid the absorption of CO_2, and kept under soda-lime tubes. The H_2SO_4 was boiled for some time to get rid of most of the volatile impurities.

The indicators used for determining the pH were thymol blue, brom phenol blue, methyl orange, methyl red, brom cresol purple, phenolsulfonephthalein, and phenolphthalein. Buffer solutions, made according to Sörensen's tables, were used as standards for comparison.

The rate is taken as the reciprocal of the time required for the change in the pH value (in the indicator tube) from 7.8 to 7.6. This time varied according to the amount of bacterial suspension used.

The temperature was kept at $21° \pm 1°$C.

Control experiments were performed with all the solutions in the absence of bacteria, and also with dead bacteria killed by boiling for one-half hour.

Experiments were carried out for 2 hours, after which time the rate of production of CO_2 remained practically constant for several hours. For this reason it was not thought necessary to include in the figures the data obtained after 2 hours. It is assumed that when the course of the reaction reached this point equilibrium was attained.

Fig. 1 shows the effect upon the rate of production of CO_2 by *Bacillus butyricus* when varying amounts of H_2SO_4 are added to the

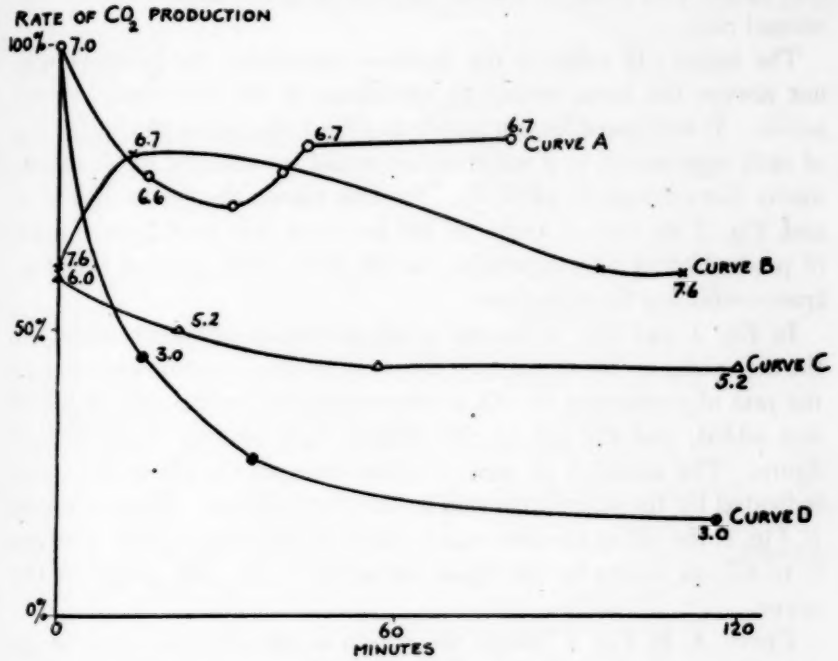

FIG. 1. Curves showing the progressive effect of acid upon the pH value and upon the rate of production of CO_2 by *B. butyricus* (expressed as per cent of the normal). The point marked zero on the abscissæ indicates the initial pH value and the rate of CO_2 production of the bacteria at the instant before acid is added. The figure attached to the first reading indicates the pH just after the addition of acid; subsequent figures indicate the pH values at the times when the readings were taken. The normal rate (which is taken as 100 per cent) represents a change in the pH value of the indicator tube in the apparatus from 7.8 to 7.6 in a number of seconds, depending upon the amount of bacterial suspension used, when the pH value of the organisms was 7.0. Each curve represents a single typical experiment.

medium containing the bacteria at different hydrogen ion concentrations.

It was found that the rate was at a maximum at pH 7; this was accordingly called 100 per cent, and arbitrarily designated as the "normal" rate. All rates were then designated as per cent of the normal. When, for example, the initial pH was 6, enough alkali was added to make the pH 7. The rate at pH 6 was then divided by the rate at pH 7 in order to express the rate at pH 6 as per cent of the normal rate.

The initial pH value of the dextrose containing the bacteria was not always the same owing to variations in the organisms themselves. It was found impracticable to adjust the pH at the beginning of each experiment to a value which would be uniform for all experiments (for example to pH 7.0). For this reason the curves in Fig. 1 and Fig. 2 do not all begin at 100 per cent, but at different rates of production of CO_2 depending on the initial pH value of the dextrose containing the organisms.

In Fig. 1 and Fig. 2 the acid or alkali respectively were added at the zero point on the abscissæ. The first reading in each case denotes the rate of production of CO_2 at the instant before the acid or alkali was added, and the pH at this instant is shown by the attached figure. The addition of acid or alkali changed the pH to the value indicated by the figure attached to the first reading. Thus in curve *B*, Fig. 1, the pH at the start was 7.6 and the addition of acid changed it to 6.7, as shown by the figure attached to the first point on the curve.

Curve *A*, in Fig. 1, shows the effects of the addition of acid to bacteria contained in a solution whose initial pH was 7.0. The pH fell at once to 6.6 then rose to 6.7. The production of CO_2 fell and rose with the change in pH value.

Curves *C* and *D* illustrate the effect of adding larger amounts of acid: in these cases the pH remains stationary after the addition of acid while the production of CO_2 falls steadily until equilibrium is attained.

Curve *B* illustrates the effect of adding acid to an alkaline suspension of bacteria (pH 7.6). The pH changed at once to 6.7 and subsequently fell. The production of CO_2 rose and fell in a characteristic manner.

Fig. 2 shows the effects upon the rate of production of CO_2 by *Bacillus butyricus* when varying amounts of NaOH are added to the medium containing the bacteria at various H ion concentrations.

Curve *A* shows the effect upon the rate of production of CO_2 when to a bacterial suspension whose pH is 7.0, sufficient NaOH is added to make the pH 8.2. There is a considerable decrease in the rate which becomes 21 per cent of the normal. This is followed by an increase in the rate as the hydrogen ion concentration returns to neutrality.

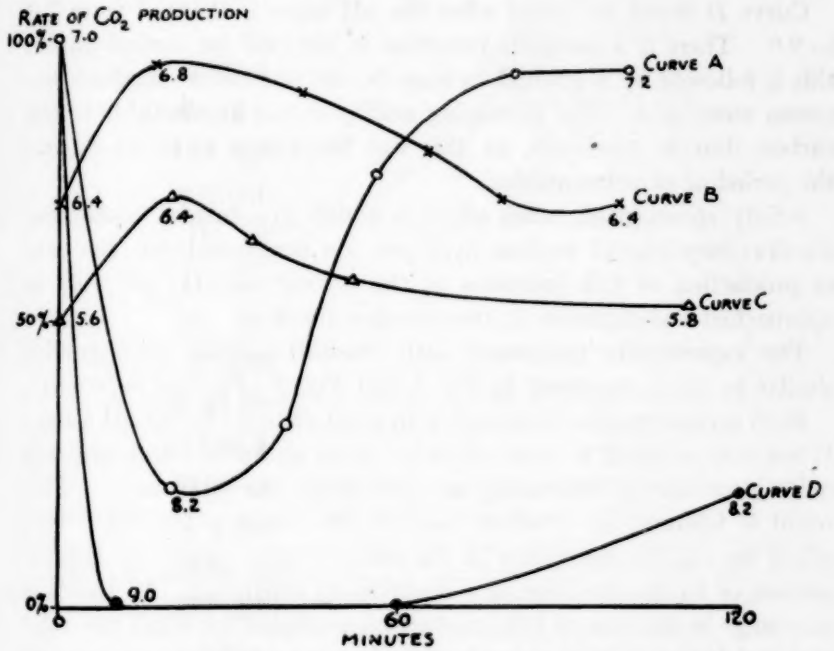

FIG. 2. Curves showing the progressive effect of alkali upon the pH value and upon the rate of production of CO_2 by *B. butyricus* (expressed as per cent of the normal). The point marked zero on the abscissæ indicates the initial pH value and the rate of CO_2 production of the bacteria at the instant before the NaOH was added. The figure attached to the first reading indicates the pH just after the addition of acid; subsequent figures indicate the pH values at the times when the readings were taken. The normal rate (which is taken as 100 per cent) represents a change in the pH value of the indicator tube in the apparatus from 7.8 to 7.6 in a number of seconds, depending upon the amount of bacterial suspension used, when the pH value of the organisms was 7.0. Each curve represents a single typical experiment.

Curve *B* shows the effect when sufficient NaOH is added to change the reaction from pH 6.3 to pH 6.8. There is an increase in the rate as the hydrogen ion concentration approaches neutrality; this is followed by a decrease. At the same time there is an increase in the hydrogen ion concentration. The final pH value in this case coincides with the initial pH value.

Curve *C* shows the effect when sufficient alkali is added to change the pH value from 5.6 to 6.4. There is an increase in the rate followed by a decrease, and the pH value becomes gradually less.

Curve *D* shows the effect when the pH value is changed from 7.0 to 9.0. There is a complete cessation in the rate for several hours; this is followed by a gradual increase in the rate as the medium becomes more acid. The increasing acidity is not attributable to the carbon dioxide produced, as this was frequently removed during the period of experimentation.

Briefly summarized, when alkali is added to solutions containing *Bacillus butyricus* of various hydrogen ion concentrations, the rate of production of CO_2 increases as the normal rate (at pH 7.0) is approached and decreases in the opposite direction.

The experiments performed with *Bacillus subtilis* gave results similar to those expressed in Fig. 1 and Fig. 2 (*Bacillus butyricus*).

Both organisms show a tendency to resist changes in the pH value. When acid or alkali is added there is always an initial effect which is quite pronounced, increasing or decreasing the pH value. This result is followed by a return towards the initial pH value. This might be due to absorption of the added acid or alkali by the organism or to the excretion of a neutralizing substance. In the case of change in the rate of CO_2 production produced by alkali the time required for recovery is considerable, but the organisms seem to be able gradually to overcome the cause of the depression in the rate.

Fig. 3 represents composite curves of a number of experiments with *Bacillus butyricus* (curve *A*) and *Bacillus subtilis* (curve *B*). The abscissæ are the various pH values, and the ordinates are the corresponding rates of CO_2 production. Each point represents the average of a number of experiments. In most cases the rate was obtained after the bacteria had been in contact with the acid or alkali from 30 to 45 minutes and had reached equilibrium (constant rate of CO_2 production).

A comparison of the two curves shows that the rate of production of CO_2 by both organisms is a function of the pH value of the medium. Below pH 5.0 *Bacillus subtilis* is very sensitive to the effects of acid, while *Bacillus butyricus* is only gradually affected as the concentration of the hydrogen ions increases. It is not possible to state, on

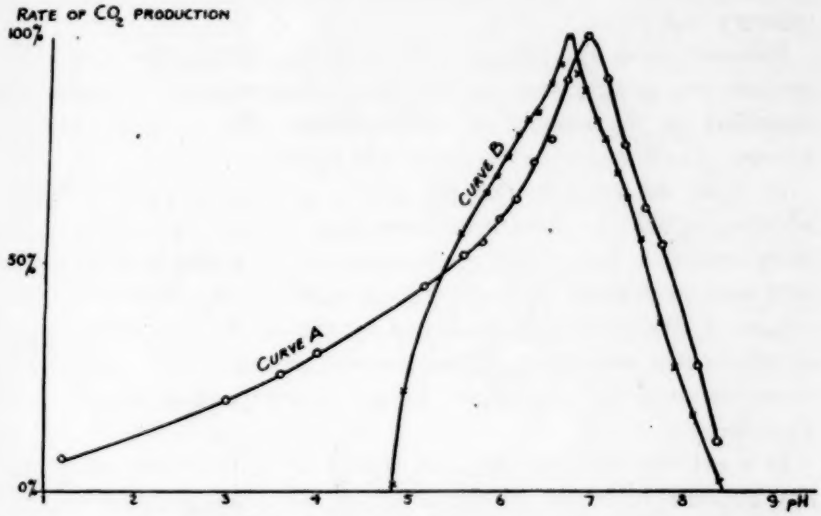

FIG. 3. Rate of production of CO_2 (expressed as per cent of the normal) *B. butyricus*, Curve *A*, and of *B. subtilis*, Curve *B*, as affected by the hydrogen ion concentrations.

The normal rate represents the maximum production of CO_2. This was obtained at pH 7.0 in the case of *B. butyricus* and at pH 6.8 in the case of *B. subtilis*. It was measured by noting the number of seconds required to change the pH value of the indicator tube from 7.8 to 7.6. The time required depended upon the amount of bacterial suspension used. Each point on the curve represents an average of several experiments. Probable error of the mean less than 2 per cent except in the last two points of the alkaline range, where it is less than 3 per cent.

the basis of these experiments, whether or not this is due, as some investigators believe, to existence of a waxy envelope in the case of the acid-fast bacteria, and to the absence of such a covering in the case of the non-acid fast bacteria.

It was found that when a small amount of acid was added to suspensions of either *Bacillus butyricus* or *Bacillus subtilis* the addition

of an equivalent amount of NaOH to the medium produced complete recovery, as shown by the production of CO_2. No recovery was obtained by washing, centrifugalizing, and rewashing the organisms in dextrose solution without the addition of NaOH. When a greater amount of acid was added, the addition of NaOH produced only partial recovery. When the medium was made extremely acid, no recovery took place.

Recovery from the effects of the addition of alkali to these organisms was spontaneous, varying in the time required for recovery according to the amount of alkali added. When an equivalent amount of acid was added recovery was hastened.

In short, decreases in the rate of CO_2 production caused by the addition of acid to these organisms were irreversible or only partially reversible (except in certain cases in which slight additions of acid were neutralized by a subsequent addition of alkali) while decreases of similar magnitude caused by the addition of alkali, were spontaneously reversible. These results are essentially similar to those obtained by Gustafson[4] in his recovery experiments with *Penicillium*.

It is evident that the effects of NaOH are not to be ascribed, to any measurable extent, to the action of Na, since previous experiments[5] have shown that when NaCl is added to *Bacillus subtilis* there is a decrease in the CO_2 production only when the total concentration of NaCl is greater than 0.15 M. In all of the above experiments the concentration of NaOH used was considerably less than this (about one-tenth as great).

It may be objected that the observed decrease in the carbon dioxide production caused by the addition of NaOH is in reality due to buffer action or to the formation of carbonates and bicarbonates as a result of the neutralization by NaOH of the CO_2 produced by the bacteria. In view of the fact that the greatest amount of alkali added in any experiment was one drop of 0.1 N NaOH it did not seem probable that this buffer action was important. The following series of experiments shows that it is negligible. The normal rate of production of CO_2 by 2 cc. of an emulsion of living bacteria was determined. A tube containing 2 cc. of distilled water was then inserted into the system so that the CO_2 coming from the bacteria

would pass through the distilled water before going into the indicator tube. After the rate of production of CO_2 under these conditions had been determined, a drop of NaOH was added to this inserted tube and its effect upon the apparent rate of CO_2 production, independent of any effect upon the bacteria, was measured. Equilibrium is very rapidly attained, since there is very little NaOH present in proportion to the amount of CO_2 produced, and since the method of stirring is very efficient. The NaOH in this tube was then replaced by distilled water and a drop of the alkali of the same concentration was placed in the tube containing the bacteria. The difference between the results in the two cases would measure the action of the NaOH upon the organisms themselves. (When this experiment was varied by using 0.75 per cent dextrose in place of distilled water the results were essentially the same.)

It was found that the addition of one drop of 0.1 N NaOH had no measurable buffer effect. This was also the case when the alkali was added to 2 cc. of an emulsion of dead bacteria in the side tube instead of to distilled water. The addition of alkali to the dead bacteria would of course simulate more nearly the buffer effects of the actual experiments.

It is not proposed to discuss at length in this paper the theoretical basis for the reactions governing the distribution of CO_2 in the apparatus. Equilibrium in the different carbonate-bicarbonate systems seems to be established almost simultaneously. Regular readings may be obtained as quickly as 5 minutes after the introduction of a solution. The hydrogen ion concentrations in the three tubes (bacterial suspension, side tube, and indicator tube) differ even when in equilibrium with the same CO_2 tension because the amount of base in the three tubes differs. They may be supposed to be in equilibrium at the beginning of a reading. After this, as the CO_2 is produced by the organisms there is an increase of CO_2 tension in the circulating air from which CO_2 is absorbed by the solutions in the tubes. The indicator tube being farthest from the source of CO_2 naturally may be expected to lag very slightly behind the side-tube in its change toward equilibrium, because the latter absorbs part of the CO_2, thus lowering the CO_2 pressure in the air which passes through it. In these experiments the side tube contained only 2 cc. of liquid, which had no measurable effect.

It appears therefore that the observed diminution of carbon dioxide production caused by the addition of NaOH to bacterial emulsions cannot be the result of any so called buffer action, but it is a true expression of the effect of an alkaline environment upon the bacteria.

SUMMARY.

1. The maximum rate of CO_2 production of *Bacillus butyricus* was found to be at a pH value of 7; of *Bacillus subtilis* at pH 6.8. If the pH value be raised or lowered there is a progressive decrease in the rate of production of CO_2.

2. Spontaneous recovery follows the addition of alkali to either organism, while addition of acid is followed by recovery only upon addition of an equivalent amount of alkali, and is not complete except when the amount of acid is very small.

THE INFLUENCE OF ELECTROLYTES ON THE SOLUTION AND PRECIPITATION OF CASEIN AND GELATIN.

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(Received for publication, September 12, 1921.)

I. INTRODUCTION.

The modern concepts of colloid chemistry originated largely from a study of the precipitation of colloids by electrolytes, and this accounts, perhaps, for the fact that the tendency to form aggregates was considered the chief characteristic of colloids in solution. As a consequence it is assumed in the text-books of colloid chemistry that the ultimate units of a colloid in solution are not isolated molecules or ions but larger aggregates of molecules or ions, the so called micella (small crumbs) of Naegeli, which are supposed to be kept in a stable solution or suspension through forces of repulsion due to the fact that they are electrically charged. The precipitation of the particles by electrolytes is ascribed to a diminution of the charge of the micella through the adsorption of the ions of the electrolyte. It was noticed by Hardy that the active or precipitating ion of the electrolyte was always that ion which had the opposite sign of charge as the colloidal particle, and Picton and Linder had noticed that the precipitating efficiency of ions increased rapidly with their valency.[1]

The experiments published by one of us have led to results which are compatible with some but not all of the assumptions just enumerated. In the first place, experiments on the osmotic pressure and the viscosity of protein solutions have made it probable that the ultimate units in certain protein solutions, such as crystalline egg albumin, are essentially isolated protein ions or molecules, though such solutions may, in a secondary way, also contain aggregates of ions or molecules.[2]

[1] Zsigmondy, R., Kolloidchemie, Leipsic, 2nd ed., 1918.

[2] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 827; 1921–22, iv, 73, 97.

It was, secondly, found that proteins combine with acids and bases according to the stoichiometrical laws of classical chemistry, and that there is neither any need nor, in fact, any room for the assumption that the ions of electrolytes are adsorbed by the protein particles in solution.[3] Thirdly, the electrical charges of protein particles which occur in such solutions are due chiefly, if not exclusively, to either the electrolytic dissociation of protein salts or to membrane potentials caused by the Donnan equilibrium.[4] Fourthly, the influence of electrolytes on the P.D., the osmotic pressure, and the viscosity of protein solutions[5] and the swelling of gels[6] can be explained quantitatively on the basis of the Donnan equilibrium. Under these circumstances it became necessary to find out whether or not the influence of electrolytes on the precipitation of proteins can be harmonized with these recent results.

All the workers who have studied the influence of electrolytes on the precipitation of colloids have come to the conclusion that there are two distinct groups of phenomena. In the one group the precipitation requires high concentrations of an electrolyte, while in the second, a low concentration of electrolyte suffices for precipitation. The difference between these two cases is so striking that it has been used for a classification of colloids. Thus, according to Zsigmondy, Noyes[7] discriminates between "colloidal solutions" and "colloidal suspensions;" the "colloidal solutions" being characterized by a high degree of viscosity, by a tendency to gelatinize, and by the fact that they are not easily precipitable by electrolytes; while the "colloidal suspensions" are, according to Noyes, characterized by a low order of viscosity, by a lack of tendency to gelatinize, and by the fact that they are easily precipitated by electrolytes.[8]

[3] Loeb, J., *Science*, 1920, lii, 449.

[4] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667.

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557, 667, 691, 827; 1921–22, iv, 73, 97.

[6] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

[7] Noyes, A. A., quoted in Zsigmondy, R., Kolloidchemie, Leipsic, 2nd ed., 1918, 29.

[8] Different authors have introduced different terms for these two groups of colloids, Perrin speaks of hydrophilic and hydrophobic colloids; Freundlich of lyophilic and lyophobic, and Wo. Ostwald of emulsoids and suspensoids.

Of all the characteristics for the two groups mentioned by Noyes, only one is essential, namely, the difference in the ease with which the two groups are precipitated by electrolytes, while the two other differences mentioned by him are only accidental. Thus a solution of crystalline egg albumin requires at ordinary temperature a high concentration of electrolyte for precipitation, although the viscosity of such a solution is low and although the solution has no tendency to gelatinize; while at a sufficiently high temperature, low concentrations of electrolytes will precipitate crystalline egg albumin though the viscosity of the solution is now high and though the solution has now a tendency to gelatinize. Hence only one of the differences mentioned in Noyes' definition remains; namely, the difference in the relative concentration required to precipitate colloids from their solution or their suspension.

The discrimination between the two types of precipitation according to whether high or low concentrations of electrolytes are required, is, however, essential and it becomes our first problem to account for this difference. We shall see that it is accompanied by another difference. When high concentrations of electrolytes are required for the precipitation of proteins the sign of charge of the protein particles is of little significance. Sulfates are much more effective for the salting out of gelatin or crystalline egg albumin from their watery solution than chlorides, regardless of whether the solution of the protein is at the isoelectric point or on the acid or alkaline side of it.

When low concentrations suffice for precipitation, the sign of charge of the protein ions becomes of paramount importance. On the acid side of the isoelectric point the active ion of the precipitating salt is the anion, while on the alkaline side it is the cation. It happens that this is also true for the Donnan effect and that in the Donnan effect also, low concentrations of electrolytes suffice for the depressing effect of a salt. This suggests the possibility that precipitation of the second group of colloids, *i.e.*, where low concentrations are required, is in some way connected with the Donnan equilibrium; and that Hardy's rule is only the consequence of this fact; while where high concentrations of electrolytes are required for precipitation the forces determining the process have no connection with the Donnan equilibrium.

In order to gain more definite information concerning the nature of the forces involved in the two cases it seemed advisable to supplement these investigations by a study of the mechanism of the solution of proteins. We have studied the mechanism of solution of granules of isoelectric casein in acid and in alkali and find that this mechanism is entirely different in the two cases. The solution of casein chloride is controlled by forces connected with the Donnan equilibrium; and it also happens that casein chloride when in solution can be precipitated by low concentrations of electrolytes; *e.g.*, M/8 NaCl. On the other hand, the solution of sodium caseinate is apparently controlled by the forces of chemical attraction between water and certain groups of the casein molecule; and it happens that very high concentrations of electrolytes, *e.g.*, $2\frac{1}{2}$ M NaCl or $3\frac{1}{4}$ M LiCl, may not be sufficient for precipitation.

II. The Precipitation and Solution of Casein Chloride.

1 per cent solutions of casein chloride of pH 2.2 were prepared in different concentrations of salts in water of about the same pH. That concentration was determined which causes an almost instantaneous complete precipitation of the protein from the originally milky solution so that the supernatant liquid became as clear as water. These concentrations were as follows:

NaCl	about M/8
$NaNO_3$	about M/8
$CaCl_2$	about M/8
Na trichloracetate	about M/16
Na_2SO_4	about M/32

Though the results are only semiquantitative, the validity of Hardy's rule and the valency effect are easily recognizable. Beside these two effects, some constitutional effects of the anion (such as the trichloracetate) may exist. It is also obvious that the concentrations of electrolytes required for instantaneous, complete precipitation of casein chloride are considerably lower than those required for the precipitation of Na caseinate from their watery solution, so that we can be sure that in the case of casein chloride we are dealing with a representative of the "suspension" group of colloids, in the sense of Noyes.

It can be shown that the solution of the casein chloride depends on forces regulated by the Donnan equilibrium and that the rule of Hardy is in this case at least only a consequence of this fact. This can be proven by microscopic observation of the mechanism of the solution of solid particles of originally isoelectric casein in solutions of acids of different concentration. It was found that the particles of casein swell in a solution of HCl, becoming more and more transparent the more they swell, and that when the swelling has reached a certain stage, the particles disappear—they are dissolved. When in the swollen stage, slight agitation may make them fall apart. T. B. Robertson had suggested such a mechanism for the solution of Na caseinate,[9] but it was found that the mechanism of solution in this latter case is different. There is no doubt, however, that the swelling of casein particles is a necessary prerequisite for the solution of casein-acid salts, since such particles are only dissolved when their swelling exceeds a definite limit.

The method of procedure was as follows: A small number of granules of isoelectric casein of the same size (going through a sieve with mesh 100 but not through a sieve with mesh 120) were put into 50 cc. of water containing different quantities of different acids and kept at 24°C. In various intervals, *i.e.*, after 15, and 60 minutes, and 6, and 24 hours, the diameter of about 15 grains was measured with a micrometer in a microscope and the average diameter calculated. The particles were not stirred, and care was taken to avoid their breaking into smaller fragments. The averages after 1 hour are plotted in Fig. 1. The abscissæ are the logarithms of the concentrations of acid of the watery solution, the ordinates are the average diameters of the particles. It is obvious that the average diameter of the particles increases at first with the increase of the concentration of the acid, reaching a maximum at about pH 2.0 of the outside solution, and with a further increase in the concentration of the acid the swelling becomes less again.

Fig. 2 gives the measurements of the same particles after 24 hours. At this time all the particles in the region of greatest solubility for HCl and for H_3PO_4, *i.e.*, between pH of the outside solution of 1.8 and 2.9, had completely dissolved and could no longer be measured.

[9] Robertson, T. B., The physical chemistry of the proteins, London, Bombay, Calcutta, and Madras, 1918, 275 ff.

Figs. 1 and 2 show another fact; namely, that the rate of swelling is not the same in different acids. It is about the same in HCl and H_3PO_4 (for the same pH) but decidedly less in HNO_3 and still less in H_2SO_4 and trichloracetic acid. It was found that the rate of solution

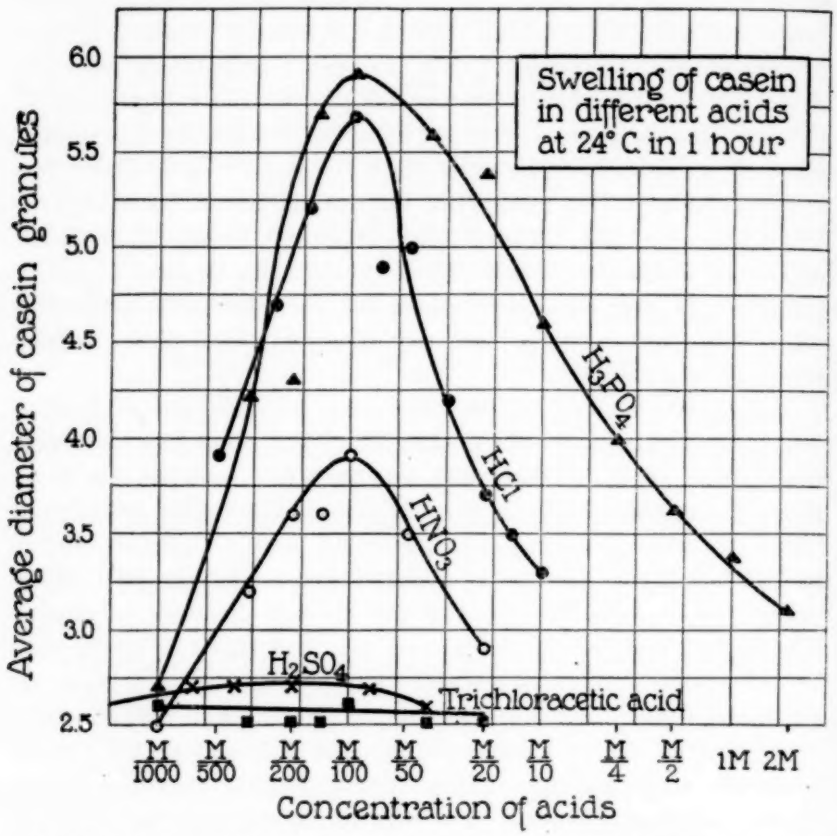

FIG. 1. Relative swelling of isoelectric granules of casein when put into acids of different concentrations, at 24°, after 1 hour. The hydrogen ion concentration of the casein particles is less than that of the outside solution on account of the Donnan equilibrium. The latter concentrations are plotted as abscissæ. The ordinates are the relative average diameters of the granules.

of casein in these different acids followed closely the rate of swelling. It took longer to dissolve casein in HNO_3 than it did in HCl (at 20°C.); and the casein was practically insoluble in H_2SO_4 and trichloracetic acid in 24 hours.

The rate of swelling is a function apparently not only of the Donnan equilibrium, but also of the force of cohesion between the particles. Procter and Wilson[6] have suggested that the rapid increase of swelling of solid gelatin with a rise in temperature is due to a corresponding

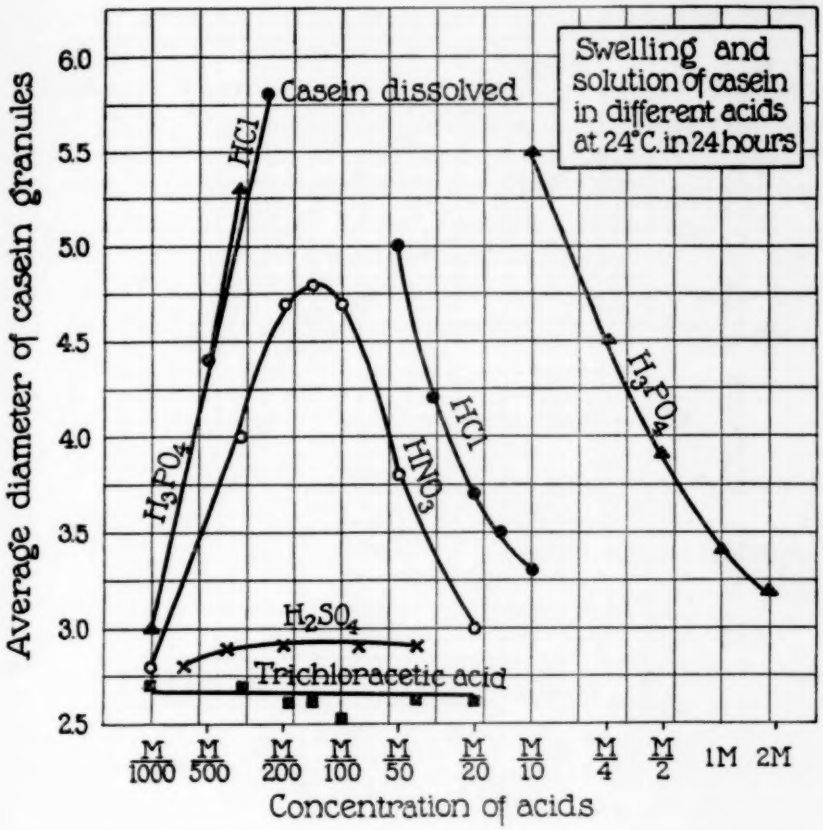

FIG. 2. Swelling of the same granules after 24 hours. Where the swelling exceeds a certain limit, between 5 and 6 in our scale, the particles are dissolved, thus showing that swelling is of importance in the mechanism of solution of casein chloride.

diminution of cohesion between the molecules of gelatin with rising temperature. The influence of the anion of gelatin-acid salts on the cohesion of the particles of a solid gel is apparently much less than the influence of the anion on the cohesion of casein particles. The

forces of cohesion in the case of casein sulfate and casein trichloracetate seem to be so great that they cannot be overcome by the osmotic pressure due to the Donnan equilibrium; and hence no swelling (and as a consequence no solution) of solid casein is possible in H_2SO_4 or trichloracetic acid.

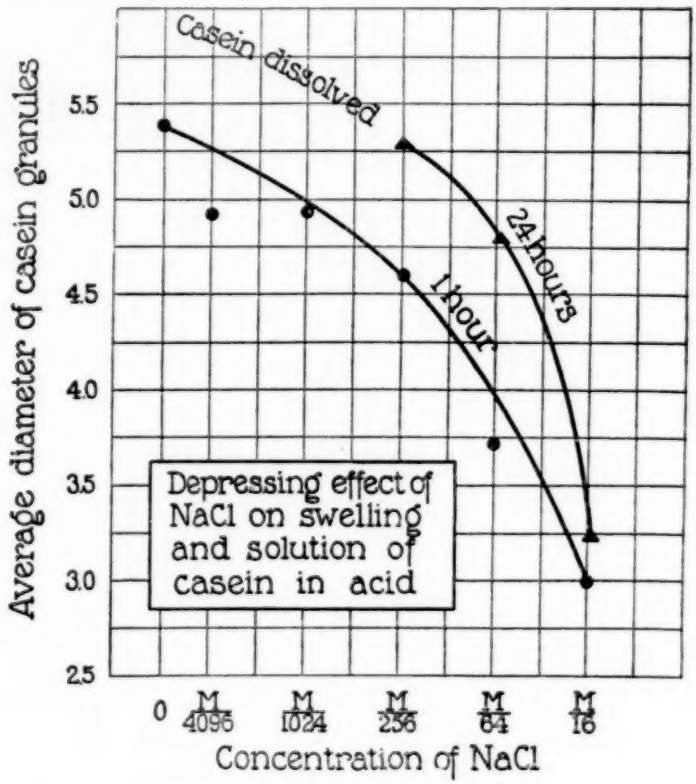

FIG. 3. Depression of swelling of casein particles in M/100 HCl by the addition of NaCl. A low concentration of acid suffices already for a noticeable depression of the swelling. The lower curve represents swelling after 1 hour; the upper curve swelling after 24 hours. Solution of the swollen particles occurs when the average diameter of the particles exceeds the value 5 to 6 in our scale.

Procter and Wilson have shown that the theory of the Donnan equilibrium explains the depressing effect of a salt on the swelling of solid gelatin.[6] Microscopic measurements of the influence of NaCl on the rate of swelling of individual grains of casein particles in M/100 HCl were made at 24°C. and the results plotted in Fig. 3.

The ordinates are the average diameters of the particles after 1 and 24 hours respectively. The abscissæ are the concentrations of NaCl. The depressing effect is similar to that found in the case of the swelling of a jelly of gelatin. After 24 hours the particles had dissolved in the NaCl solutions of a concentration of below M/256, but not in concentrations of NaCl higher than M/256.

That the solution of casein chloride is thus regulated to a large extent by the Donnan effect was ascertained also by measurements of the quantity of casein chloride dissolved at 20°C. at various pH of the solution. 1 gm. of isoelectric powdered casein was put into 100 cc. of solutions of HCl of different concentration and kept in these solutions in one case for 1 hour, in a second case for 22 hours. The mass was then poured into graduated cylinders and the undissolved part was allowed to settle to the bottom for 2 and for 6 hours respec-

TABLE I.

Amount of Casein Dissolved at 20°C in HCl of Different pH.

pH	4.36	3.32	3.11	2.97	2.94	2.84	2.75	2.64	2.53	2.36	2.18	2.06	1.87	1.66	1.50	1.40
Mg. dissolved after 1 hour	42	55	86	249	265			348	408		547	538	401	366	272	219
Mg. dissolved after 22 hours	102	133	164	267	342	459	536	634	646	733	788	779	710	528	374	300

tively at 20°C. The supernatant liquid was removed and the sediment dried over night in an oven at about 100°C. Table I gives the result. The dry weight of 1 gm. of isoelectric casein was found to be 0.870 gm. and this weight diminished by the dry weight of the sediment was the amount dissolved. Table I shows that, the rate of solution increases with diminishing pH from 4.4 to 2.18 where the solubility of casein chloride is a maximum; with a further decline in pH the solubility diminishes again. This is in agreement with the Donnan effect.

In a similar way the depressing effect of NaCl on the rate of solution of casein chloride was ascertained. Solutions of 12.5 cc. of 0.1 N HCl in 100 cc. and containing 1 gm. of powdered, originally isoelectric casein were prepared in 0, M/2048, M/1024, to M/4 NaCl. The pH of a solution of 1 gm. casein in 100 cc. containing 12.5 cc. of 0.1 N HCl was 2.12 and this pH was the same in all solutions made up in NaCl.

The solution was kept at 20° for 16 hours and then allowed to settle for 24 hours at 20° in 100 cc. graduate cylinders. The dry weight of the sediment was determined and this weight when deducted from the dry weight of 1 gm. isoelectric casein, namely, 0.870 gm., was the amount that had gone into solution after a correction was made for the free NaCl held in 2 cc. solution which was arbitrarily assumed not to have been removed. Though this latter correction was somewhat arbitrary, it could have caused a noticeable error only when the concentration of the salt solution exceeded M/64. For the solutions of M/64 and below this error was negligible. Table II gives the number of milligrams of casein which had gone into solution.

TABLE II.

	Concentration of NaCl.					
	M/2048	M/1024	M/512	M/256	M/128	M/64
Mg. dissolved.....	714	685	665	615	449	282

The main fact is that a slight increase in the concentration of NaCl causes already a noticeable drop in the rate of solution. Thus M/1024 NaCl causes already a noticeable diminution in the solubility of a 1 per cent solution of casein chloride of pH 2.12 at 24°C.

These observations then indicate that the solution of solid particles of casein chloride is brought about by the ultimate elements being forced apart mechanically through the process of swelling. The force acting in this swelling is the hydrostatic pressure of the water which is forced into the interstices of the solid particles by the osmotic pressure in the interstices between the casein ions. Procter and Wilson have shown that the application of Donnan's theory of membrane equilibrium accounts quanitatively for this swelling on the assumption that swelling is caused by the excess of the osmotic pressure inside the gel over that of the surrounding solution. As soon as the osmotic pressure in the gel exceeds the forces of cohesion between the casein ions of the gel the casein ions constituting the gel are separated.

The question then arises: How can the Donnan effect stabilize the particles of casein chloride in solution, and how can we explain the

precipitating effect of low concentrations of neutral salts? Let us assume that the ultimate particles in a solution of casein chloride of pH 2.2 are, (*a*) isolated casein ions, (*b*) isolated casein molecules, and (*c*) small casein aggregates or micella. The Donnan equilibrium furnishes two kinds of forces preventing that degree of coalescence of these particles which is required for precipitation; namely, the osmotic pressure and the membrane potentials. When isolated protein ions collide and remain attached to form a micellum, a Donnan equilibrium is established between the nascent micellum and the surrounding solution. The Donnan equilibrium demands that there be a higher concentration of electrolytes inside than outside and this difference in osmotic pressure leads to water being attracted into the micellum. The increase in hydrostatic pressure will force the protein molecules apart again and thus tends to prevent the formation of the micellum. Moreover, if micella exist in the casein chloride solution (aside from isolated casein ions and molecules) the coalescence of different micella into larger aggregates must be prevented by the potential difference between the micella and the surrounding solution. J. A. Wilson suggested in 1916[10] that the source of the charges might be the Donnan equilibrium, and one of us has recently shown that these potential differences between a gel and the surrounding solution, which Donnan's theory demands and which Wilson postulated, actually exist.[11] As a consequence of this P.D., the micella are charged and must repel each other according to the charge. This charge caused by the Donnan equilibrium is a minimum at the isoelectric point, rises with increasing hydrogen ion concentration, reaching a maximum, and diminishes again with a further increase in hydrogen ion concentration as shown in a preceding paper. The osmotic pressure and charge are also diminished by the addition of salt.

Hardy's rule that only that ion of a neutral salt is active in precipitation which has the opposite sign of charge as the colloidal ion, and that the efficiency of the ion increases with the valency is simply the expression of the Donnan effect, as is also the fact that very low concentrations of electrolytes suffice for precipitation. The reader will notice that it is unnecessary to assume that the ions

[10] Wilson, J. A., *J. Am. Chem. Soc.*, 1916, xxxviii, 1982.
[11] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557, 667.

are adsorbed by the casein and that this adsorption of ions annihilates the electrical charges on the particles of casein.

Emulsions of oil drops in water are prevented from coalescing by their electrical charges (and not by osmotic forces). The investigations of Beutner[12] have shown that the P.D. at the boundary of water and substances immiscible in water is determined by an unequal distribution of crystalloidal ions between the two phases, and the writer ventures to suggest that this distribution may be regulated by Donnan's theory, owing to the fact that, *e.g.*, the oleic acid anion in oil cannot diffuse into the water. The similarity of the behavior of the P.D. between oil and water and between solid gelatin and water is rather striking. The depression of the charge on the particles through the addition of salt can in both cases be explained without the adsorption hypothesis.

III. The Precipitation and Solution of Na Caseinate.

When we prepare solutions of Na caseinate of pH 11.0 containing 1 gm. of originally isoelectric casein in 100 cc., we notice that it requires enormous concentrations of NaCl or LiCl to precipitate the casein. NaCl as concentrated as $2\frac{1}{2}$ M and LiCl as concentrated as $3\frac{1}{4}$ M were not able to precipitate Na caseinate from its solution, while M/8 NaCl was sufficient for instantaneous and complete precipitation of casein chloride from its solution. Hence Na caseinate belongs to the other group of colloidal solutions; namely, those which require high concentrations of electrolytes.

When a grain of isoelectric casein is put into a solution of NaOH Na caseinate is formed at the surface of the solid granule. This dissolves in water, as any crystalloidal substance does (*i.e.*, by the forces of residual valency), except for the accidental fact that the solution of Na caseinate seems to lower the surface tension of the watery solution at the interface, as is suggested by the rather violent spreading of the Na caseinate solution at the interface, which can be observed under the microscope. As a result projecting particles at the surface of the granule are torn away from the surface of the solid casein, collect-

[12] Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

ing at a short distance, while the originally rough surface of the grain of casein becomes smooth. These phenomena of solution accompanied by spreading occur soon all over the surface. The small particles of casein carried away in the spreading motion are gradually completely dissolved and the result is a solution of Na caseinate of a high osmotic pressure, indicating that the solution consists to a large extent of isolated protein ions.

The mechanism of the solution of casein in NaOH is essentially that of the solution of a crystal except for the purely accidental and secondary phenomenon that the surface tension of the Na caseinate solution is considerably lower than that of a watery NaOH solution, and that thus the orderly process of solution noticeable in the case of crystals is disturbed by phenomena of spreading. The mechanism of solution of grains of solid casein in NaOH resembles the solution of sodium oleate in water, which is also accompanied by a lowering of the surface tension at the interface. This interference of the phenomena of spreading in the process of solution of Na caseinate may account for the fact that Robertson[9] was unable to confirm in this case the solution theory of Noyes and Whitney,[13] according to which there exists at the boundary of crystals and water a film of solution which is always saturated, the velocity of solution being determined by the rate of diffusion of the dissolved crystalloid out of this film into the body of the fluid. It is natural that this theory should be masked whenever the process of solution is accompanied by phenomena of spreading due to a lowering of surface tension at the interface. The forces dragging the particles of Na caseinate into solution are to all appearances those forces of residual valency between certain groups of the molecule of Na caseinate and water which also cause the solution of crystalloids in water, while the forces dragging the particles of casein chloride into water are forces which have their origin in the Donnan equilibrium. A quotation from Langmuir will illustrate the rôle which active groups of a complex molecule may play in the process of true solution.

"Acetic acid is readily soluble in water because the COOH group has a strong secondary valency by which it combines with water. Oleic acid is not soluble because the affinity of the hydrocarbon chains for water is less than their affinity

[13] Noyes, A. A., and Whitney, W. R., *Z. physik. Chem.*, 1897, xxiii, 689.

for each other. When oleic acid is placed on water the acid spreads upon the water because by so doing *the COOH can dissolve in the water* without separating the hydrocarbon chains from each other.

"When the surface on which the acid spreads is sufficiently large the double bond in the hydrocarbon chain is also drawn onto the water surface, so that the area occupied is much greater than in the case of the saturated fatty acids.

"Oils which do not contain active groups, as for example pure paraffin oil, do not spread upon the surface of water."[14]

That the solution of isoelectric casein in NaOH has no connection with the Donnan equilibrium is also evidenced by the fact that there is no point in the concentration of NaOH where further increase in the concentration of NaOH lowers the rate of solution, as would be the case if the Donnan equilibrium influenced this phenomenon.

The stability of solutions of this kind is guaranteed by the forces of chemical attraction between certain groups of the molecule of Na caseinate and water; and the precipitation by electrolytes is due to a diminution of these forces.

IV. Precipitation and Solution of Gelatin in Water.

Solutions of gelatin in water require enormous concentrations of salts for precipitation and it is very probable that the forces causing solution are the forces of residual valency discussed in the case of the solution of Na caseinate. These cases have no connection with the Donnan equilibrium, and this is borne out by the well known fact that solutions of gelatin are always more readily salted out by sulfates than by chlorides, regardless of the pH of the gelatin solution. This is illustrated by Table III.

0.8 per cent solutions of gelatin were prepared at three different pH; namely, 4.7 (isoelectric gelatin), 3.8 (gelatin chloride), and 6.4 to 7.0 (Na gelatinate). The purpose was to find the molecular concentration of different salts, namely, $(NH_4)_2SO_4$, Na_2SO_4, $MgSO_4$, KCl, and $MgCl_2$, required for precipitation. Table III shows that regardless of the pH the sulfates are better precipitants than the chlorides. There is, therefore, no definite relation between the sign of charge of the colloidal particles and of the precipitating ion.

[14] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1850.

Precipitation experiments give no insight into the reason why sulfates are superior to chlorides in salting out; and an attempt was made to get an answer by experiments on the solution of gelatin. Such experiments gave the result that salts increase the solubility of isoelectric gelatin, and the more, the higher their concentration until finally a concentration is reached where the reverse effect is noticed. This reversal occurs at a comparatively low concentration in the case of the sulfates, while in the case of salts like $CaCl_2$ or $MgCl_2$ it does not seem to occur at all in the limit of the concentrations tried. The experiments were carried on at a temperature of 35°C.

Powdered gelatin of not too small a size of grain (going through sieve 30 but not through sieve 60) was rendered isoelectric in the way described in previous articles, and 0.8 gm. was put into 100 cc. of each of a series of solutions of NaCl, $CaCl_2$, or Na_2SO_4 varying in concentration from M/4096 to 2 M. The suspensions of the powdered gelatin were frequently stirred and the time required to practically completely dissolve all the grains of powdered gelatin in suspension at 35°C. was measured. We consider this time the reciprocal of the solubility. The ordinates in Fig. 4 are the solution times (*i.e.*, the reciprocal of the solubility) of isoelectric gelatin, and the abscissæ are the molecular concentrations of the salt used. It is obvious that NaCl and still more $CaCl_2$ increase the solubility (or diminish the solution time) of isoelectric gelatin in water, and the more, the higher the concentration of the salts. There exists a striking discontinuity in the Na_2SO_4 curve. As long as the concentration of Na_2SO_4 is below M/32 it increases the solubility of gelatin, and the more so, the higher the concentration. When, however, the concentration

TABLE III.

Minimal Molar Concentrations Required to Precipitate 0.8 Per Cent Solutions of Gelatin.

pH of gelatin solution.	Approximate molecular concentration of salt required for precipitation.				
	$(NH_4)_2SO_4$	Na_2SO_4	$MgSO_4$	KCl	$MgCl_2$
4.7	15/16 M	6/8 M	10/8 M	>3 M	>3 M
3.8 (gelatin chloride)	13/16 M	5/8 M	7/8 M	3 M	>3 M
6.4 to 7.0 (Na gelatinate)	16/16 M	7/8 M	9/8 M	>3 M	>3 M

of Na_2SO_4 is above M/32, a further increase in the concentration of Na_2SO_4 diminishes the solubility of gelatin, and the more so, the higher the concentration of Na_2SO_4. $(NH_4)_2SO_4$ acts in the same way. We now understand why it is that we cannot precipitate solutions of isoelectric gelatin with KCl or $MgCl_2$ in concentrations

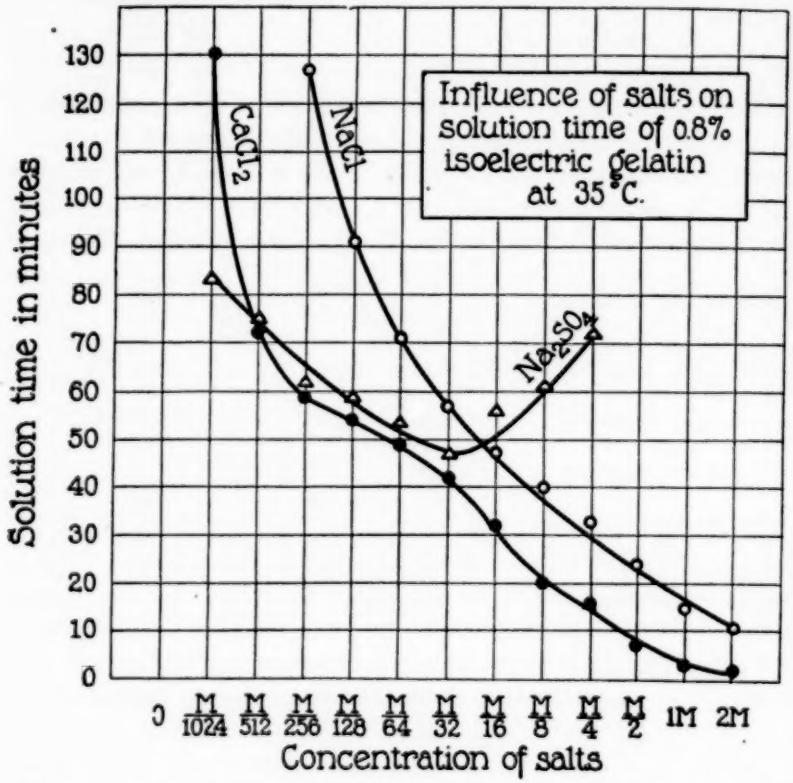

FIG. 4. Influence of salts on the time required to completely dissolve 0.8 gm. of powdered isoelectric gelatin in 100 cc. of salt solution of different concentrations at 35°C. Abscissæ are the concentrations of the salt; ordinates, the time required for complete solution.

up to 3 M (see Table III) while we can precipitate such solutions with sulfates but only at concentrations above M/2; since our curve shows that at such high concentrations of sulfates the solubility of isoelectric gelatin in Na_2SO_4 becomes less than in pure water.

While isoelectric gelatin is only sparingly soluble, gelatin salts

are highly soluble. 0.8 gm. of powdered gelatin of pH of about 3.3 dissolves very rapidly in 100 cc. HCl of the same pH at 35°C. The addition of NaCl or $CaCl_2$ no longer increases the solubility, except when the concentration of $CaCl_2$ becomes greater than M/16. Na_2SO_4

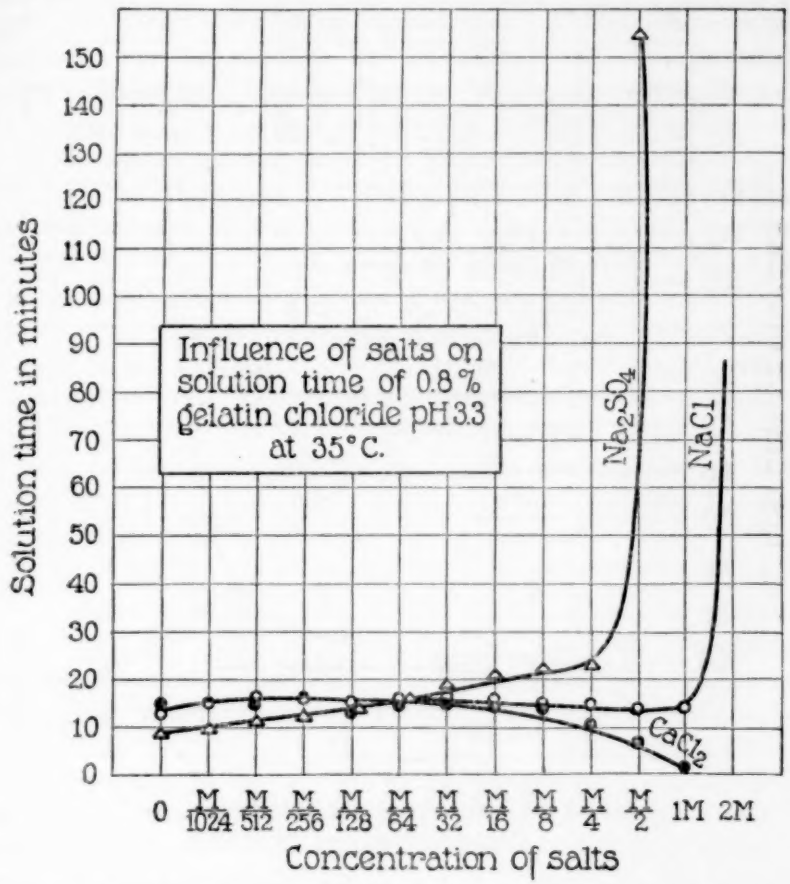

FIG. 5. Influence of salts on time required to dissolve 0.8 gm. of powdered gelatin chloride of pH 3.3 at 35°C.

abruptly diminishes the solubility at a concentration above M/4 and NaCl above a concentration of 1 M (Fig. 5).

Fig. 6 shows the influence of the three salts on the solution time of Na gelatinate of pH 10.5. Na_2SO_4 diminishes the solubility abruptly

at a concentration above M/8, while both NaCl and $CaCl_2$ increase the solubility of Na gelatinate, NaCl in concentrations above M/2, and $CaCl_2$ in concentrations above M/16.

In all three cases, therefore, is the solubility of gelatin diminished by sulfates, but only exceptionally by chlorides. This explains the

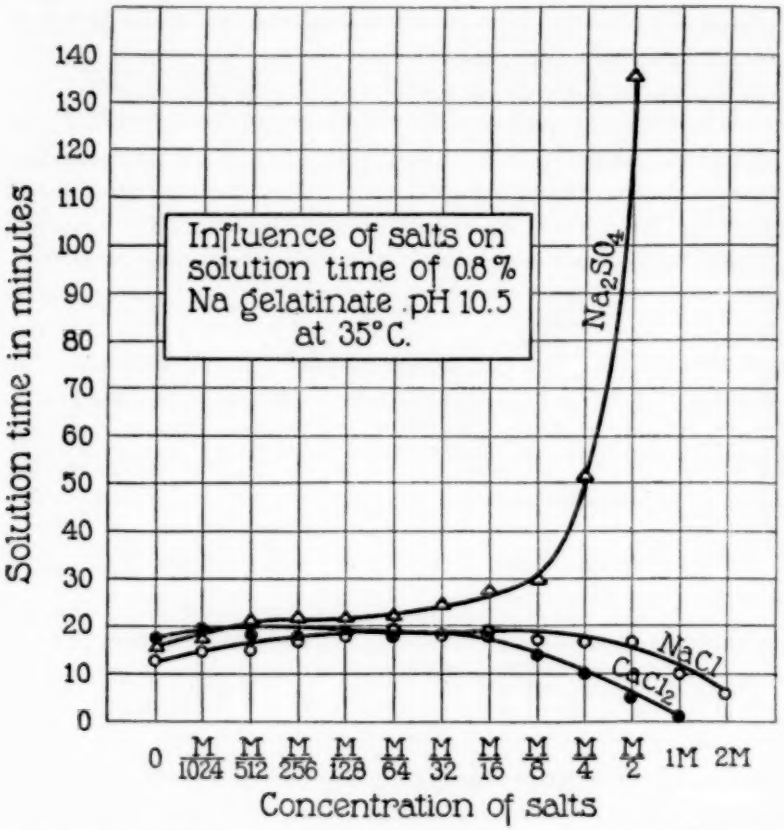

FIG. 6. Influence of salts on the time required to dissolve 0.8 gm. of powdered Na gelatinate of pH 10.5 at 35°C.

results contained in Table III.

We have seen that addition of little acid to isoelectric gelatin increases the osmotic pressure, viscosity, P.D., and swelling with increasing concentration, while beyond a certain pH the addition of more acid has a depressing effect. This is characteristic of the

Donnan effect. It was of interest to find out whether such a maximum followed by a drop existed in the influence of acid on the solubility of gelatin, but this is not the case at least between pH 4.7 and 1.0. Measurements of the dry weight of gelatin dissolved in a certain time at different pH, showed that the amount of gelatin dissolved increases with the hydrogen ion concentration. This corroborates the conclusion that the solution (and precipitation) of gelatin in water is not influenced by forces governed by the Donnan equilibrium and does therefore not show the characteristics of colloidal behavior.

These experiments also contradict the suggestion that the solution of solid gelatin is necessarily preceded by swelling and that swelling and solution are continuous processes. The contradiction lies in the fact that swelling in acid reaches a maximum at pH of about 3.0 and then diminishes upon further increase in hydrogen ion concentration, while the rate of solution of solid gelatin granules continues to increase steadily when the hydrogen ion concentration increases beyond pH of 3.0. The mechanism of swelling and the mechanism of solution of solid gelatin in water are determined by forces of an entirely different character; the swelling by osmotic pressure, and the solution by the secondary valency forces responsible for the solution of crystalloids.

V. Solubility and Viscosity of Gelatin Solutions.

We assumed in a preceding paper that the increase in the viscosity of gelatin solutions on standing is due to the gradual formation of larger aggregates from originally isolated gelatin molecules or gelatin ions. When we melt a solid gel of gelatin by rapidly heating it to 45°C. and cooling it rapidly to 20° there is produced a true solution containing isolated gelatin molecules or gelatin ions side by side with submicroscopic pieces of solid jelly. On standing two opposite processes are constantly going on in such a mixture; *i.e.*, the solution of these aggregates into isolated molecules or ions and the reverse process; namely, that of the formation of new aggregates by the union of formerly isolated gelatin molecules or gelatin ions. When the velocity of aggregate formation prevails over the velocity of the solution of aggregates, the viscosity of the gelatin solution will increase on standing; when the velocity of solution of aggregates

prevails over the velocity of formation, the viscosity of the gelatin solution will diminish on standing. All agencies which accelerate the rate of solution of solid gelatin should counteract the aggregate formation and the rise of viscosity of a gelatin solution on standing; while all agencies which diminish the rate of solution of solid gelatin should increase the rise of viscosity of the gelatin solution on standing.

We have seen that powdered isoelectric gelatin is dissolved the more rapidly, the more HCl we add, and that there is no maximum followed by a drop when a certain hydrogen ion concentration is exceeded. Hence we should expect that the lower the pH of a gelatin solution containing the same concentration of originally isoelectric gelatin, the smaller the increase of the viscosity of the solution on standing. In a preceding paper we have already given curves showing that this is the case and that there is no Donnan effect noticeable in this case.[15]

We have seen in Fig. 5 that Na_2SO_4 commences to diminish noticeably the rate of solution of solid gelatin chloride as soon as the concentration of Na_2SO_4 exceeds M/64, while $CaCl_2$ commences to have the opposite effect as soon as the concentration of $CaCl_2$ exceeds M/4.

We prepared gelatin chloride solutions of pH 3.4 containing 1 gm. of originally isoelectric gelatin in 100 cc. solution. The solutions were made up in various concentrations of Na_2SO_4 and $CaCl_2$. The solutions were rapidly heated to 45°, rapidly cooled to 20°C., and kept at this temperature for 1 hour. The time of outflow of the solution through a viscometer was measured immediately and in intervals of 5 or 10 minutes. The time of outflow of water through the viscometer at 20° was 61 seconds.

The viscosity of a gelatin chloride solution of pH 3.4 rises gradually but very slowly (uppermost curve in Fig. 7) and the rate of increase of viscosity on standing is not materially altered in M/512 Na_2SO_4 and only little in M/128 Na_2SO_4. In M/32 Na_2SO_4 the viscosity increases more rapidly on standing, in M/8 Na_2SO_4 still more rapidly, and in M/2 Na_2SO_4 very sharply. This is exactly what we should expect since the Na_2SO_4 causes a diminution of the rate of solution of gelatin chloride as soon as the concentration of

[15] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 97.

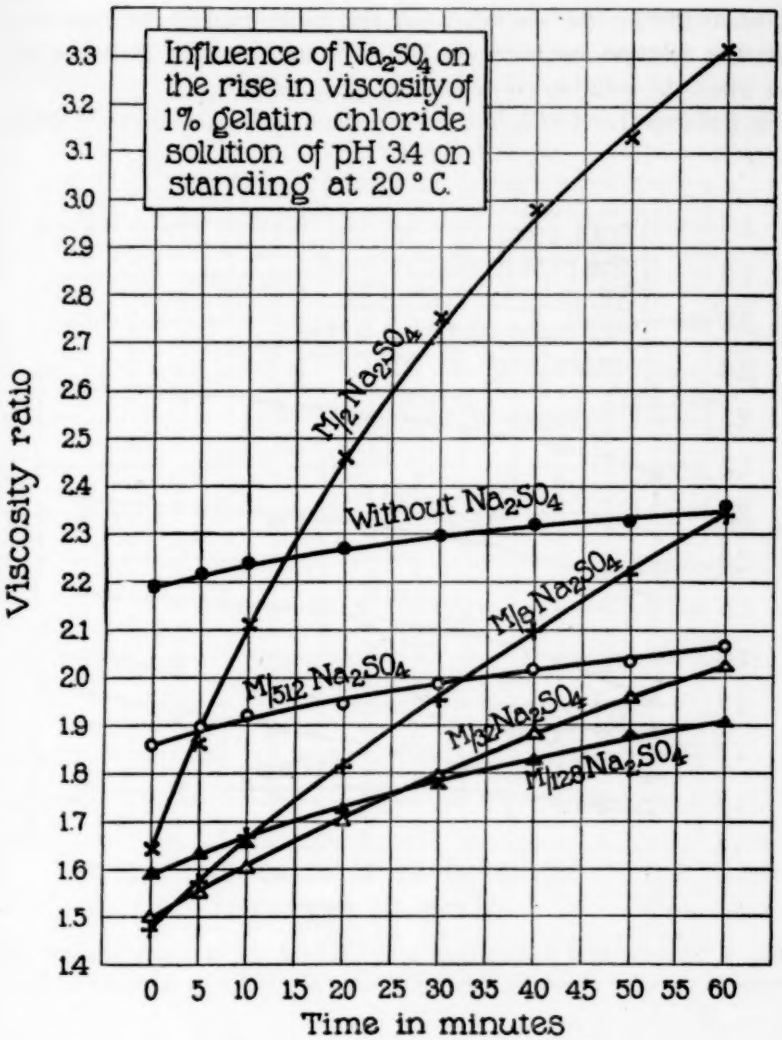
Influence of Na_2SO_4 on the rise in viscosity of 1% gelatin chloride solution of pH 3.4 on standing at 20°C.
Viscosity ratio
Time in minutes
M/2 Na_2SO_4
Without Na_2SO_4
M/8 Na_2SO_4
M/512 Na_2SO_4
M/32 Na_2SO_4
M/128 Na_2SO_4

FIG. 7. Showing that concentrations of Na_2SO_4 of M/32 and above cause an increase in the viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

Na_2SO_4 is above M/64. In such solutions the rate of solution of micella will be less and less, and since new micella are constantly formed at 20°C., the viscosity will rise more rapidly on standing when the solution contains Na_2SO_4 in concentrations above M/64 than when the solution contains no or less Na_2SO_4.

Fig. 8 shows that $CaCl_2$ in concentrations up to M/8 does not alter

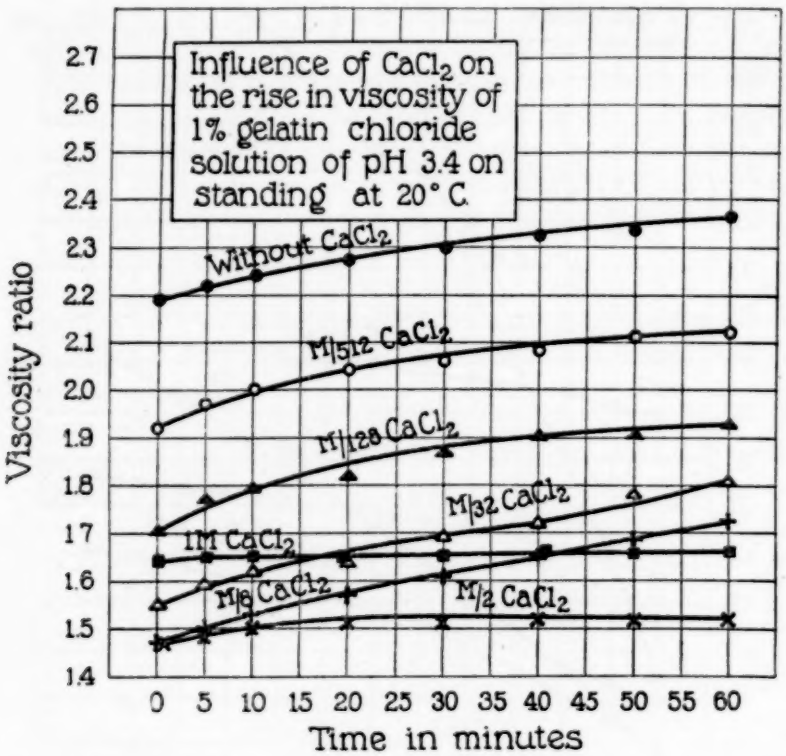

FIG. 8. Showing that concentrations of $CaCl_2$ of M/2 or above prevent the increase in viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

the increase in viscosity of gelatin chloride solution on standing but that the viscosity of gelatin chloride of pH 3.4 does no longer increase on standing when the concentration of $CaCl_2$ is M/2 or 1 M. In this concentration $CaCl_2$ causes a slight increase in the rate of solution of gelatin chloride.

NaCl causes no change in the rate of solution of gelatin chloride as long as the concentration of NaCl does not exceed 1 M. Above this concentration it causes coagulation and the viscosity can no longer be measured. Hence NaCl in concentrations up to 1 M should

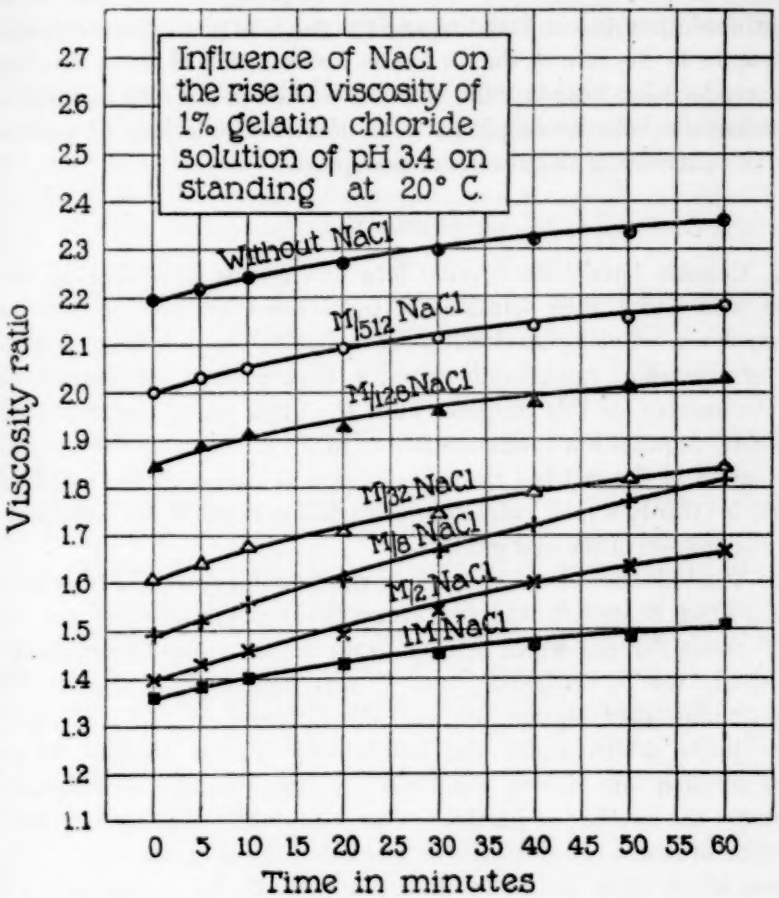

FIG. 9. Showing that NaCl solutions up to a concentration of 1 M have no effect on the increase in viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

not alter the rate of increase of viscosity of gelatin chloride solutions on standing. Fig. 9 shows that this is correct.

These experiments corroborate the idea that gelatin solutions

are true solutions consisting of isolated gelatin ions and molecules which may contain, in addition, submicroscopic aggregates of gelatin molecules and ions. The formation of new micella and a solution of these already formed occur constantly. When the rate of formation of such micella exceeds the rate of the solution, the viscosity of a gelatin solution rises on standing and the rise is in proportion with the difference in the rate of the formation and solution of these micella. The reader must bear in mind that the micella in the case of gelatin solutions are submicroscopic particles of a reversible jelly of gelatin and not particles of an irreversible precipitate.

SUMMARY.

1. Colloids have been divided into two groups according to the ease with which their solutions or suspensions are precipitated by electrolytes. One group (hydrophilic colloids), *e.g.*, solutions of gelatin or crystalline egg albumin in water, requires high concentrations of electrolytes for this purpose, while the other group (hydrophobic colloids) requires low concentrations. In the latter group the precipitating ion of the salt has the opposite sign of charge as the colloidal particle (Hardy's rule), while no such relation exists in the precipitation of colloids of the first group.

2. The influence of electrolytes on the solubility of solid Na caseinate, which belongs to the first group (hydrophilic colloids), and of solid casein chloride which belongs to the second group (hydrophobic colloids), was investigated and it was found that the forces determining the solution are entirely different in the two cases. The forces which cause the hydrophobic casein chloride to go into solution are forces regulated by the Donnan equilibrium; namely, the swelling of particles. As soon as the swelling of a solid particle of casein chloride exceeds a certain limit it is dissolved. The forces which cause the hydrophilic Na caseinate to go into solution are of a different character and may be those of residual valency. Swelling plays no rôle in this case, and the solubility of Na caseinate is not regulated by the Donnan equilibrium.

3. The stability of solutions of casein chloride (requiring low concentrations of electrolytes for precipitation) is due, first, to the osmotic pressure generated through the Donnan equilibrium between

the casein ions tending to form an aggregate, whereby the protein ions of the nascent micellum are forced apart again; and second, to the potential difference between the surface of a micellum and the surrounding solution (also regulated by the Donnan equilibrium) which prevents the further coalescence of micella already formed. This latter consequence of the Donnan effect had already been suggested by J. A. Wilson.

4. The precipitation of this group of hydrophobic colloids by salts is due to the diminution or annihilation of the osmotic pressure and the P.D. just discussed. Since low concentrations of electrolytes suffice for the depression of the swelling and P.D. of the micella, it is clear why low concentrations of electrolytes suffice for the precipitation of hydrophobic colloids, such as casein chloride.

5. This also explains why only that ion of the precipitating salt is active in the precipitation of hydrophobic colloids which has the opposite sign of charge as the colloidal ion, since this is always the case in the Donnan effect. Hardy's rule is, therefore, at least in the precipitation of casein chloride, only a consequence of the Donnan effect.

6. For the salting out of hydrophilic colloids, like gelatin, from watery solution, sulfates are more efficient than chlorides regardless of the pH of the gelatin solution. Solution experiments lead to the result that while $CaCl_2$ or NaCl increase the solubility of isoelectric gelatin in water, and the more, the higher the concentration of the salt, Na_2SO_4 increases the solubility of isoelectric gelatin in low concentrations, but when the concentration of Na_2SO_4 exceeds M/32 it diminishes the solubility of isoelectric gelatin the more, the higher the concentration. The reason for this difference in the action of the two salts is not yet clear.

7. There is neither any necessity nor any room for the assumption that the precipitation of proteins is due to the adsorption of the ions of the precipitating salt by the colloid.

THE ORIGIN OF THE POTENTIAL DIFFERENCES RESPONSIBLE FOR ANOMALOUS OSMOSIS.

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(Received for publication, September 19, 1921.)

I. Potential Differences in Collodion Membranes Coated with Protein.

In a series of papers[1] the writer has published the results of experiments showing the peculiar influence of electrolytes on the rate of diffusion of water through collodion membranes. When a collodion membrane separates a solution of an electrolyte (of not too high a concentration) from pure warer, the water diffuses from the side of pure water to the solution side, but the initial rate at which this diffusion occurs does not obey van't Hoff's law. The initial rate of diffusion was measured by the rise of level of liquid in a glass tube inserted through a rubber stopper in a closed collodion bag containing the solution, the collodion bag dipping into a beaker filled with pure water. When the membrane was merely a collodion membrane, the following rules expressed the influence of electrolytes on the initial rate of diffusion of water through the membrane from the water side to the side of the solution.

1. Water diffuses into the solution of an electrolyte through a collodion membrane as if the particles of water were positively charged and as if they were attracted by the anion and repelled by the cation of the electrolyte in solution with a force increasing with the valency.

2. In the case of certain electrolytes the "attracting force" of the anion for water increases at first more rapidly with increasing concentration than the "repelling force" of the cation, until a point is reached where with a further increase in concentration the "repelling force" of the cation increases more rapidly than the "attractive force" of the anion. Finally, a concentration is reached where the

[1] Loeb, J., *Science*, 1921, liii, 77; *J. Gen. Physiol.*, 1918–19, i, 717; 1919–20, ii, 173, 387, 577.

"attracting" and "repelling" forces balance each other and from then on the "attractive" force of the solution increases with the concentration, apparently in accordance with van't Hoff's law.

When, however, the collodion membrane has received a coating of a protein, *e.g.*, gelatin, the hydrogen ion concentration influences the

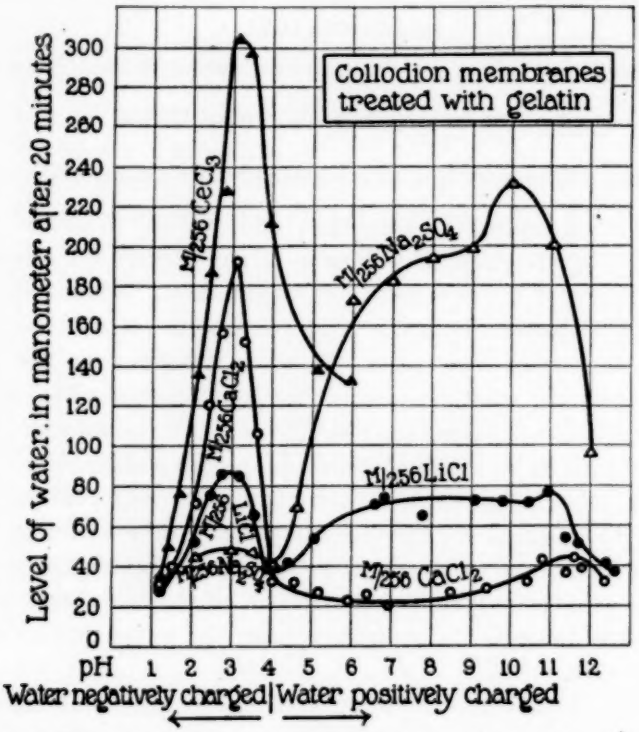

FIG. 1. Influence of hydrogen ion concentration on the initial rate of diffusion of liquid from a solution of HNO_3 or NaOH respectively through a gelatin-coated membrane of collodion into a M/256 solution of LiCl, $CaCl_2$, $CeCl_3$, or Na_2SO_4 of the same hydrogen ion concentration. Abscissæ are the initial pH; ordinates, the rise of level of liquid in manometer connected with the salt solution, after 20 minutes.

initial rate of diffusion of water from pure water into the solution in a still more complicated way (Fig. 1). The procedure in these experiments was as follows: Both the outside solution as well as the inside solution had the same hydrogen ion concentration,

containing the same concentration of HNO_3 or NaOH respectively. In addition the inside solution (*i.e.*, the solution inside the collodion bag which was connected with the manometer) contained a M/256 solution of one of the following four salts: $CeCl_3$, $CaCl_2$, LiCl, and Na_2SO_4. The "attraction" of these M/256 salt solutions for water was therefore tested at different hydrogen ion concentrations. It is obvious from Fig. 1 that the "attractive" force of one and the same salt solution for water (measured by the initial rate of diffusion of water from the outside into the salt solution) varied considerably with the hydrogen ion concentration of the solution. (The hydrogen ion concentration is expressed by Sörensen's logarithmic symbol pH; *i.e.*, the log of the concentration with the minus sign omitted). The abscissæ in the curve are the initial pH of the solutions, while the ordinates are the rise in level of the watery liquid in the glass tubes after 20 minutes at 24°C.

Fig. 1 shows that somewhere between pH 4.0 and 5.0 a reversal of the sign of charge of the water particles occurs. At pH below 4.0 the water is negatively charged, at pH above 5.0, it is positively charged. This change coincides with a change in the nature of the charge of the gelatin ion. At a pH of 4.0 or below the gelatin forms gelatin nitrate (in the presence of HNO_3) and hence the gelatin ion is a cation. The water, being negative, has the opposite sign of charge as the gelatin ion. At a pH of 5.0 or above the gelatin forms metal gelatinate, Na gelatinate, Ca gelatinate, etc., and the gelatin ion is negatively charged. The water, being positively charged, has again the opposite charge as the gelatin ion. The exact turning point, the isoelectric point of gelatin, is at a pH 4.7. In a preceding paper[2] the writer has been able to show that if other proteins than gelatin are used to give the collodion membrane a coating of a protein, the reversal of the sign of charge of the water varies with the isoelectric point of the protein used.

If we return to Fig. 1 we notice that where the water is negatively charged it is "attracted" by the cation of the salt used and "repelled" by the anion with a force increasing with the valency of the ion. The attractive force increases from Li to Ca to Ce, and diminishes from

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 577.

Cl to SO_4. Where the water is positively charged the reverse order of efficiency exists.

The fact that the same concentration of the salts, namely, M/256, was used, has no essential influence on the results. Similar results were obtained when the salt solutions had the osmotic pressure of a M/64 solution of cane sugar.

Those who have studied phenomena of abnormal osmosis through membranes, Girard,[3] Bernstein,[4] Bartell and his collaborators,[5] and Freundlich[6] have reached the conclusion that potential differences on the opposite sides of the membrane are responsible for these anomalies in diffusion. The fact that the sign of charge varies in these experiments with the sign of charge of the protein ion gives a favorable point of attack for the investigation of the origin of the potential differences. In order to simplify the experiments they were confined to pH from 4.6 to pH 1.9; *i.e.*, to that region where the water diffuses as if it were negatively electrified. The method of procedure was as follows. M/256 solutions of one of the four salts mentioned were prepared in different concentrations of HNO_3 and put into collodion bags of about 50 cc. volume, which were lined by a film of gelatin on the inside. These bags were closed with a perforated rubber stopper through which was pushed a glass tube with a diameter of about 2 mm. to indicate the rise of liquid. The collodion bags were put into beakers containing 350 cc. of the same concentration of HNO_3 as that inside the bag. Fig. 2 gives the rise of the level of liquid in the manometer in one set of experiments after 1 hour at 24°C. The abscissæ are the initial pH of the liquid which was the same inside and outside, the ordinates are the rise of the level of liquid after 1 hour. It is obvious in this case that the curves have a minimum near the isoelectric point of gelatin, that the rate of diffusion of water into the solution rises

[3] Girard, P., *Compt. rend. Acad.*, 1908, cxlvi, 927; 1909, cxlviii, 1047, 1186; 1910, cl, 1446; 1911, cliii, 401; La pression osmotique et le méchanisme de l'osmose, Publications de la Société de Chimie-physique, Paris, 1912.

[4] Bernstein, J., Elektrobiologie, Braunschweig, 1912.

[5] Bartell, F. E., *J. Am. Chem. Soc.*, 1914, xxxvi, 646. Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1036. Bartell, F. E., and Madison, O. E., *J. Physical Chem.*, 1920, xxiv, 593.

[6] Freundlich, H., *Kolloid. Z.*, 1916, xviii, 11.

with the increasing hydrogen ion concentration reaching a maximum at pH 3.6 or 3.0 respectively, and that the rate of diffusion falls again with a further increase in the hydrogen ion concentration. M/256 Na_2SO_4 shows little "attraction" for water, and M/128 cane sugar practically none.

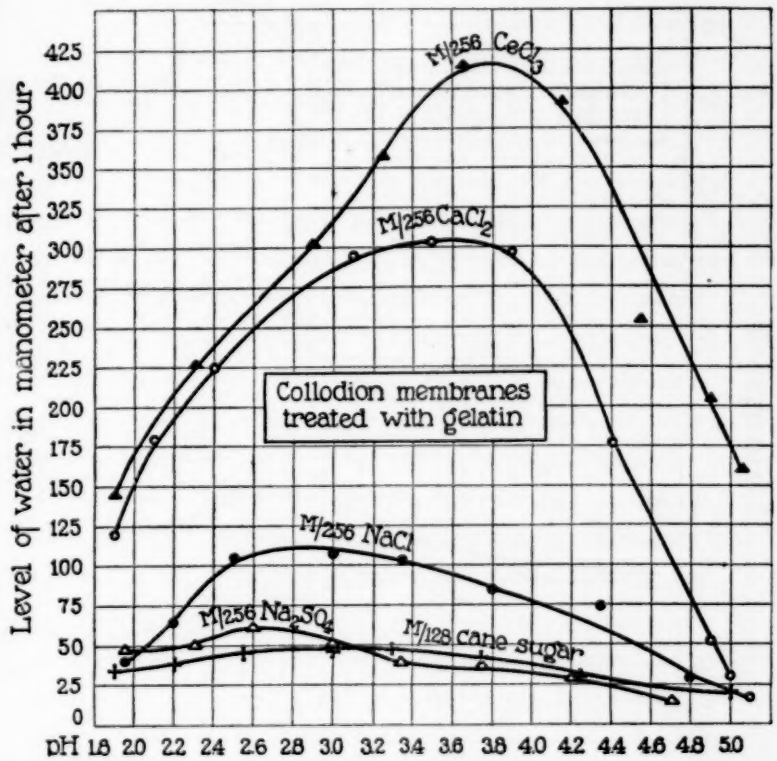

FIG. 2. Influence of hydrogen ion concentration on initial rate of diffusion of liquid from a solution of HNO_3 through a gelatin-coated collodion membrane into a M/128 solution of cane sugar, M/256 NaCl, $CaCl_2$, $CeCl_3$, or Na_2SO_4 of the same hydrogen ion concentration. Abscissæ are the pH; ordinates, rise of level of liquid in manometer connected with the salt solution, after 1 hour. Water negatively charged.

The writer has recently measured the potential differences between the inside salt solution and the outside solution at the end of an hour with the aid of a Compton electrometer (calomel electrodes with

saturated KCl solution) and obtained a set of curves presented in Fig. 3. At this time some of the salt had diffused from the inside into the outside solution. The P.D. curves in Fig. 3 resemble the osmotic curves in Fig. 2 sufficiently to suspect a connection

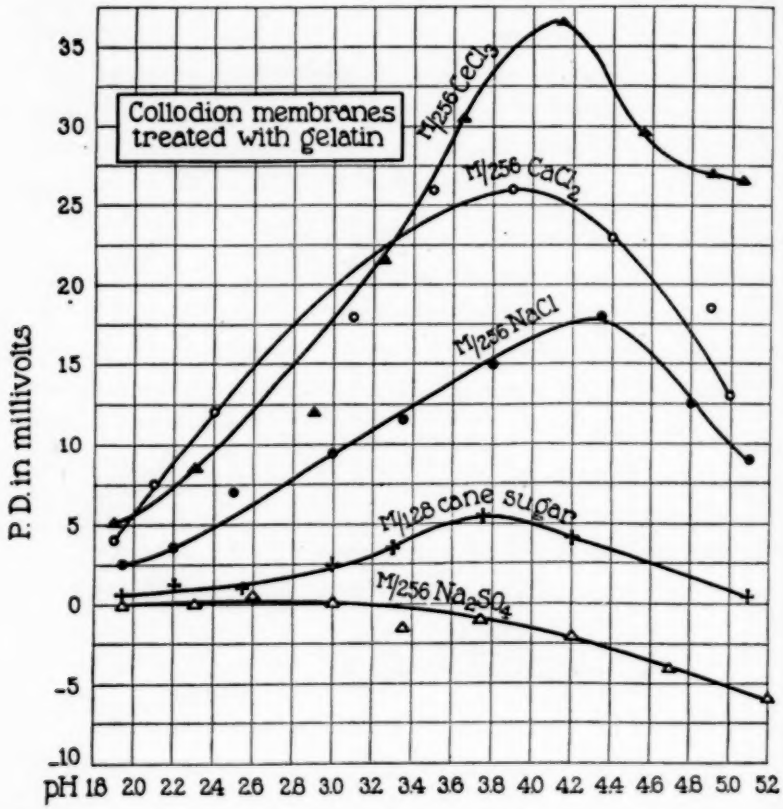

FIG. 3. Potential difference between salt solutions and external solution in preceding experiment after 1 hour of diffusion. Abscissæ are the pH at beginning of experiment; ordinates, P.D. at the end of the experiment. Attention is called to a certain similarity of the curves in Figs. 2 and 3.

between the two, so that we may assume that the P.D. between the opposite sides of the membrane is the main driving force which (for low concentrations of electrolytes) moves the water from the water side to the solution side of the membrane.

The next question was: What is the origin of the P.D.? It occurred to the writer that a measurement of the hydrogen ion concentration inside and outside after 1 hour might possibly throw a light on this problem. The results of these measurements are plotted in Fig. 4. The reader will remember that at the beginning the hydrogen ion concentration was the same inside and outside. Yet after 1 hour

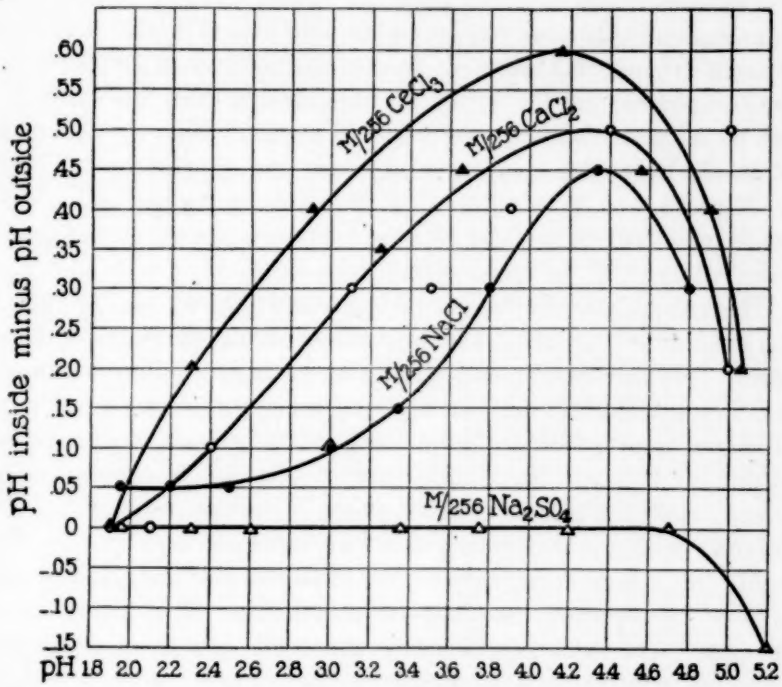

FIG. 4. Abscissæ are the initial pH; ordinates, the values (pH inside minus pH outside) in preceding experiment. Attention is called to the similarity of the curves in Figs. 3 and 4.

(and in fact much earlier) it is obvious that some of the acid has diffused from the inside into the outside solution so that the hydrogen ion concentration is higher outside than inside the collodion bag containing the salt solution.

This then leads to the result that when we separate identical acid solutions by a collodion membrane coated with a film of gelatin, and put inside the collodion bag a solution of a neutral salt with a mono-

valent anion (*e.g.*, Cl), acid is driven from the side where the salt is added to the side originally free from salt. This difference in the pH inside minus pH outside gives rise to a potential difference which was discussed in a preceding paper.[7] In this paper it was shown that when we separate a gelatin chloride solution from a solution of HCl (without gelatin) both having at the beginning the same hydrogen ion concentration, acid is forced from the gelatin solution into the outside solution. The same happens when solid jelly of gelatin chloride is separated from a HCl solution. In this case there arises a P.D. between the two phases; and the writer has been able to show that this P.D. can be calculated with a good degree of accuracy from the difference of the hydrogen ion concentration inside and outside, on the basis of Nernst's well known formula. By multiplying the value (pH inside minus pH outside) by 58 we get the value for the P.D. actually observed at 18°C. in terms of millivolts.[7]

This unequal distribution of acid inside and outside is due to a peculiar membrane equilibrium the theory of which was developed by Donnan.[8] Our new experiments show that such an equilibrium condition is produced also between a gelatin membrane and a solution of HCl free from gelatin. The coating of gelatin on the collodion membrane behaves like a solid jelly of gelatin, the gelatin being transformed into gelatin chloride when in contact with HCl. The Donnan equilibrium demands that the concentration of free HCl inside the gelatin membrane be less than the concentration of free HCl of the solution bounding the membrane. This gives rise to the P.D. The value of pH inside minus pH outside is diminished when a neutral salt is added—in accordance with Donnan's theory—and the fact that a salt is added in our diffusion experiments on one side of the membrane but not on the other is the cause of the fact that the pH of the acid solution containing the salt solution (inside solution) becomes higher than the pH of the solution containing no or less salt (outside solution). The reader will find the experiments proving this in the writer's paper referred to.[7]

[7] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667.

[8] Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

A comparison of Figs. 3 and 4 shows that the two sets of curves, that for the P.D. (Fig. 3) and for the values pH inside minus pH outside (Fig. 4) when plotted over the initial pH as abscissæ, have certain features in common. Both sets of curves have a minimum at or near the isoelectric point of gelatin (pH 4.7). They both rise to a maximum at or not far from pH 4.2, and then drop again when the initial pH of the solution drops further. This parallelism suggests that this form of the P.D. curve is determined by the differences in the hydrogen ion concentration produced on the opposite sides of the membrane in conformity with Donnan's theory of membrane equilibrium.

II. Potential Differences in Collodion Membranes Free from Protein.

The experiments just described were repeated with collodion membranes which had not been treated with a protein. The difference between the P.D. in this case (Fig. 5) and the P.D. observed in the case of collodion membranes coated with a film of gelatin (Fig. 3) is striking. The method of experimentation was the same in the case of the collodion bags free from gelatin as in the experiments of the preceding chapter. The initial concentration of HNO_3 was always the same inside and outside. The inside solution, however, contained in addition to the acid a solution of M/256 of one of the four salts, $CeCl_3$, $CaCl_2$, NaCl, and Na_2SO_4. After 1 hour the P.D. inside and outside was measured.

We notice that the P.D. is no longer a minimum at or near pH = 4.0 and we can therefore be certain that this feature of the curves in Fig. 3 was due to the gelatin. The curves in Figs. 3 and 5 have, however, one feature in common; namely, that the P.D. increases with the increasing valency of the cation and diminishes with the increasing valency of the anion. This feature is, therefore, not a specific function of the film of gelatin.

Since the water is positively charged even in the presence of acid when the membrane consists of collodion free from protein, it is to be expected that only the Na_2SO_4 solution should attract the water, while no such attraction should occur in the case of M/256 $CeCl_3$ or M/256 $CaCl_2$. Fig. 6 shows that this is true.

The question then arises: How can we account for this valency effect of the ion with the same sign of charge as that of the particle of water? The answer seems to be that we are dealing here with diffusion potentials which are independent of the membrane. This was proved

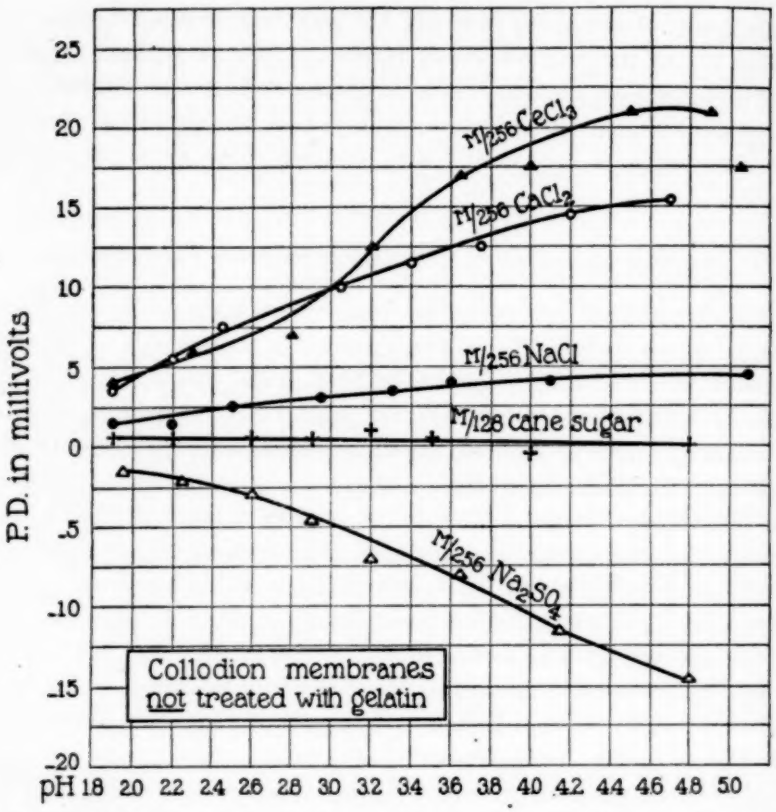

FIG. 5. Influence of neutral salts on the P.D. across collodion membranes not treated with gelatin. Otherwise same experiment as in Fig. 3. Notice difference between curves in Figs. 5 and 3, revealing the share of the protein in the origin of the P.D.

in the following way. Two beakers were filled with identical solutions of HNO_3, the HNO_3 solution in one of the beakers being dissolved in M/256 $CeCl_3$ or $CaCl_2$ or NaCl or Na_2SO_4, while the HNO_3 in the other was dissolved in pure water. The pH was identical in

the two solutions. They were connected by a bent glass tube, filled with a pure HNO_3 solution, of the same pH as that in the two beakers. Into each beaker was dipped a calomel electrode with saturated KCl solution and the diffusion potential was ascertained with the aid of a Compton electrometer. Fig. 7 gives the P.D. A comparison between Figs. 5 and 7 shows that the curves representing the P.D. in the two cases are very similar.

If we now return to a discussion of the curves in Figs. 1 and 2, representing the influence of the pH on the attraction of a M/256 solution of a neutral salt for water through a collodion membrane impregnated

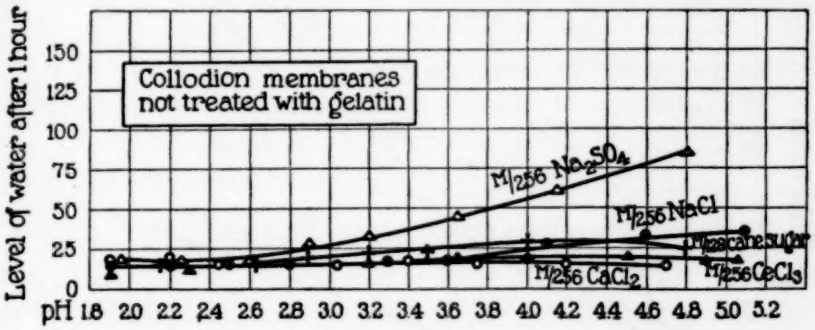

FIG. 6. Influence of M/128 cane sugar, M/256 NaCl, $CaCl_2$, $CeCl_3$, and Na_2SO_4 on the initial rate of diffusion of water through collodion membranes not treated with gelatin. Otherwise the experiment is the same as in Fig. 2. Abscissæ are the pH; ordinates, rise of level of water in salt solution after 1 hour. Only Na_2SO_4 attracts water to a noticeable amount since the latter is positively charged when diffusing through the membrane.

with gelatin, we can say that these curves resemble the curves in Fig. 3 for the P.D. on the opposite sides of the membrane.

Figs. 4 and 7 show that the P.D. curves in Fig. 3 have a double source. One is connected with the influence of the gelatin layer on the unequal distribution of the acid on the opposite sides of the membrane, which finds its explanation probably in the Donnan effect. The other source of the P.D. seems to be the diffusion potentials as shown in Fig. 7.

These diffusion potentials which exist regardless of the presence or absence of a membrane and regardless of the nature of the membrane seem to be responsible for the fact that the ions with opposite sign

of charge as that of the water "attract" the water with a force increasing with the valency of the ion, while the Donnan effect seems to be

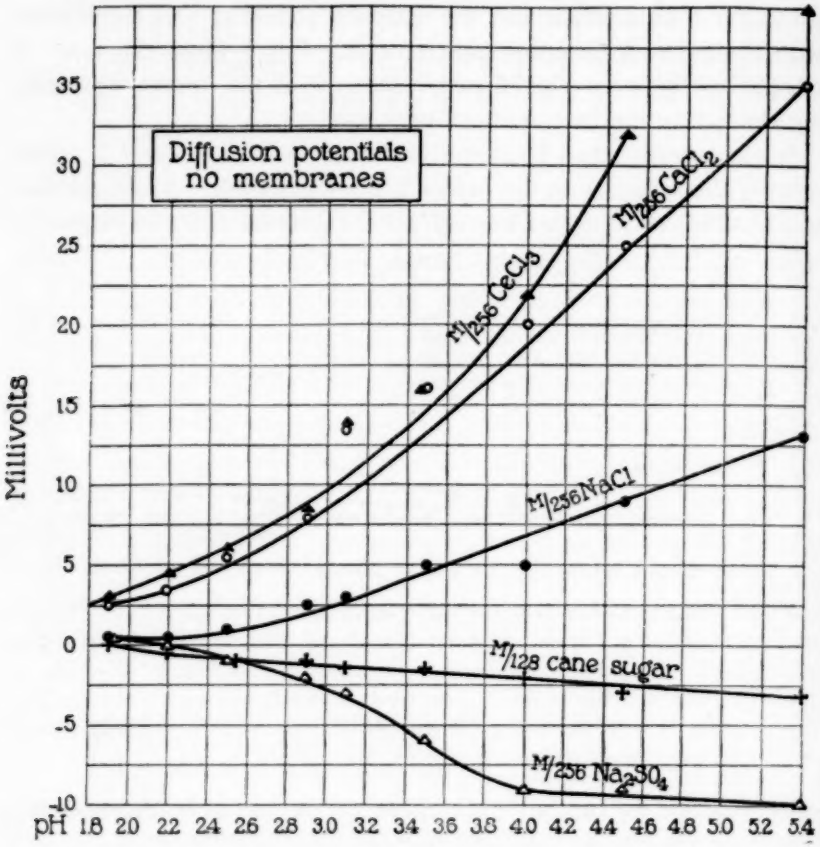

FIG. 7. Diffusion potentials between solutions of M/128 cane sugar, M/256 NaCl, $CaCl_2$, $CeCl_3$, and Na_2SO_4 in various concentrations of HNO_3 and solutions free from these salts but of the same concentrations of HNO_3. The salt solutions and the solutions free from salt had always the same pH. Abscissæ are the pH; ordinates, diffusion potentials. Attention is called to the resemblance between the curves in Fig. 7 and those in Fig. 5.

responsible for the fact that when the collodion membrane is impregnated with a protein the rate of diffusion of the negatively charged water is a minimum at the isoelectric point of the protein, increasing

with a diminishing pH until a maximum is reached at a pH of about 4.2, and diminishing with a further diminution of the pH.

These experiments then lead to the result that the anomalous osmosis discussed in this paper is determined by a potential difference between the opposite sides of the membrane, and that this P.D. seems to have a double source; namely, the Donnan equilibrium and diffusion potentials.

In addition to these two sources of P.D. between the opposite sides of the membrane there may be others, as, *e.g.*, the Coehn effect due to the difference in the dielectric constant between liquid and membrane. If such a P.D. plays a rôle in our experiments it can be only of minor importance.

The experiments suggest the possibility that the establishment of a Donnan equilibrium between membrane and solution is one of the factors determining the Helmholtzian electrical double layer, at least in the conditions of our experiments.

SUMMARY AND CONCLUSION.

1. Collodion bags coated with gelatin on the inside were filled with a M/256 solution of neutral salt (*e.g.*, NaCl, $CaCl_2$, $CeCl_3$, or Na_2SO_4) made up in various concentrations of HNO_3 (varying from N/50,000 to N/100). Each collodion bag was put into an HNO_3 solution of the same concentration as that inside the bag but containing no salt. In this case water diffuses from the outside solution (containing no salt) into the inside solution (containing the salt) with a relative initial velocity which can be expressed by the following rules: (*a*) Water diffuses into the salt solution as if the particles of water were negatively charged and as if they were attracted by the cation and repelled by the anion of the salt with a force increasing with the valency of the ion. (*b*) The initial rate of the diffusion of water is a minimum at the hydrogen ion concentration of about N/50,000 HCl (pH 4.7, which is the point at which gelatin is not ionized), rises with increasing hydrogen ion concentration until it reaches a maximum and then diminishes again with a further rise in the initial hydrogen ion concentration.

2. The potential differences between the salt solution and the outside solution (originally free from salt) were measured after the

diffusion had been going on for 1 hour; and when these values were plotted as ordinates over the original pH as abscissæ, the curves obtained were found to be similar to the osmotic rate curves. This confirms the view expressed by Girard, Bernstein, Bartell, and Freundlich that these cases of anomalous osmosis are in reality cases of electrical endosmose where the driving force is a P.D. between the opposite sides of the membrane.

3. The question arose as to the origin of these P. D. and it was found that the P.D. has apparently a double origin. Certain features of the P.D. curve, such as the rise and fall with varying pH, seem to be the consequence of a Donnan equilibrium which leads to some of the free HNO_3 being forced from the solution containing salt into the outside solution containing no (or less) salt. This difference of the concentration of HNO_3, on the opposite sides of the membrane leads to a P.D. which in conformity with Nernst's theory of concentration cells should be equal to 58 $\times$ (pH inside minus pH outside) millivolts at 18°C. The curves of the values of (pH inside minus pH outside) when plotted as ordinates over the original pH as abscissæ lead to curves resembling those for the P. D. in regard to location of minimum and maximum.

4. A second source of the P.D. seems to be diffusion potentials, which exist even if no membranes are present and which seem to be responsible for the fact that the rate of diffusion of negatively charged water into the salt solution increases with the valency of the cation and diminishes with the valency of the anion of the salt.

5. The experiments suggest the possibility that the establishment of a Donnan equilibrium between membrane and solution is one of the factors determining the Helmholtzian electrical double layer, at least in the conditions of our experiments.